

WATER
WATER
WATER
WATER
WATER
WATER
WATER
WATER
WATER
WATER
WATER

PROJECT COMPLETION
REPORT NO. 345X

The Influence of
Suspended Microscopic
Substances on the
Metabolic Activities
of Microorganisms
Responsible for
Biological
Enrichment of Water

By
Robert M. Pfister
1971

United States Department
of the Interior

CONTRACT NO.
A-006-Ohio
AA-14-01-0001-1855



FINAL REPORT

THE INFLUENCE OF SUSPENDED MICROSCOPIC SUBSTANCES ON THE METABOLIC
ACTIVITIES OF MICROORGANISMS RESPONSIBLE FOR
BIOLOGICAL ENRICHMENT OF WATER

ROBERT M. PFISTER
THE OHIO STATE UNIVERSITY

WATER RESOURCES CENTER
THE OHIO STATE UNIVERSITY
COLUMBUS, OHIO 43210

345X-A-006-OHIO

AA-14-01-0001-1855

1971

TABLE OF CONTENTS

PART A "Influence of Aldrin and Varied Oxygen Concentrations on a Lake Erie Heterotrophic Bacterium: Electron Microscopic Examination," by R. S. Kennedy and R. M. Pfister.

Abstract	i
Introduction	1
Materials and Methods	2
Results	4
Discussion	8
Literature Cited	13

PART B "Influence of Aldrin and Varied Oxygen Concentrations on a Lake Erie Heterotrophic Bacterium: Growth Characteristics and Biosynthetic Patterns," by R. S. Kennedy and R. M. Pfister .

Abstract	i
Introduction	1
Materials and Methods	2
Results	5
Literature Cited	16

PART C "Microparticulates: Isolation from Water and Identification of Associated Chlorinated Pesticides," by R. M. Pfister, P. R. Dugan, and J. I. Frea.

Abstract	i
Introduction	1
References	7

PART D "Aldrin: Removal from Lake Water by Flocculent Bacteria," by W. O. Leshniowsky, P. R. Dugan, R. M. Pfister, J. I. Frea, and C. I. Randles

Abstract	i
Introduction	1
References	8

PART E "Particulate Fractions in Water and the Relationship to Aquatic Microflora," by R. M. Pfister, P. R. Dugan, J. I. Frea, and F. T. Stone.

Abstract	i
Introduction	1
Methods and Results	2
Discussion	10
References	15

Table of Contents, Continued

PART F "Chlorinated Hydrocarbon, Microparticulate Effects on Microorganisms
Isolated from Lake Erie," by R. M. Pfister, J. I. Frea, P. R. Dugan,
C. I. Randles, K. Zaebst, J. Duchene, T. McNair, and R. Kennedy.

Abstract	i
Introduction	1
Methods and Results	2
Discussion	10
References	15

LIST OF TABLES

	<u>Page</u>
PART A	
TABLE 1. Ultrastructural Differences Observed in Cells Grown Under Different Environmental Conditions.	11
PART B	
TABLE 1. Viable Cell Numbers and Final Optical Densities of Cultures in the Stationary Phase and the Generation Times of the Organisms in the Log Phase of Growth..	13
TABLE 2. Average Cell Size, Cell Dry Weight, Total Cell Numbers of <u>Pseudomonas sp</u> at Different Stages of the Growth Cycle and Grown Under Different Conditions .	14
TABLE 3. Concentration of PHB in Stationary Phase Cells Grown Under Different Environmental Conditions.	15
PART C	
TABLE 1. Nanograms of Chlorinated Hydrocarbon Pesticides Per Liter of Lake Water Associated With Each Fraction of Particulates. Samples 79 and 75 Show the Qualitative Presence or Absence of Chlorinated Pesticides in Each Extracted Fraction of Two Other Samples Taken at Different Times of the Year.	4
PART D	
TABLE 1. Results of an Experiment Showing the Effect on Microbial Growth by Two Particulate Fractions and Their Supernatants. The Bars of the Histogram Represent the Relative Growth Measured Turbidimetrically as Compared to a Growth Control (No Fraction). Each Bar Has Been Corrected for the Effects of Turbidity Caused by Addition of the Particulates. . .	9
TABLE 2. Results of an Experiment Showing the Effects of the Addition of a 0.3 Micron and Larger Particulate Fraction on the Growth of <u>Streptomyces sp.</u> and <u>Micro-monospora sp.</u> The Graph Shows Increase in Relative Biomass as Measured by Increase in DNA Content Throughout the Experiment. The Arrow Indicates the Concentration of This Fraction in the Lake	11

This report constitutes the final report for the allotment grant #A 006 OHIO. This project was attempted as a seed project to start a larger program concerning microbial ecology and pollution. The investigators associated with this grant have used it as a base upon which to build a more complex program. We have generated a sincere interest in the area of ecology and pollution with a large number of students and technicians as well as solidifying our own positions. At this time we have had four masters degrees and one Ph.D. degree awarded, all of which received partial or total support from this grant. There will be several other degrees awarded in the near future which can be attributed to this effort as well (see list of thesis attached).

There have been five scientific presentations at national or regional meetings of interested people. (See list of presentations). We have submitted several abstracts for future meetings during 1971 at this time.

There have been four completed and accepted publications and we have submitted two more to the Journal of Bacteriology for review. These two papers have been included as part of this final report and are herewith attached to it. The titles of the papers as yet unaccepted are: "Influence of Aldrin and Varied Oxygen Concentrations on a Lake Erie Heterotrophic Bacterium: Growth Characteristics and Biosynthetic Patterns," and "Influence of Aldrin and Varied Oxygen Concentrations on a Lake Erie Heterotrophic Bacterium: Electron Microscope Examination"

During these studies we have attempted to examine and experiment with particulate suspended colloidal material in the water column of Lake Erie on a physical, chemical, and biological basis. We have characterized various inorganic and detrital fractions using differential and gradient centrifugation in conjunction with electron microscopy. Various fractions of particulate material have been experimented with as to their ability to influence biological activity. Data from our studies have shown that particulates from Lake Erie are comprised of substances having a variety of densities and sizes and that fractions can be separated which exert an influence on the growth and metabolism of microorganisms isolated from Lake Erie. Specific microparticulates in a lake such as Lake Erie will alter the microecology and have substantial effect on the macroecology. We have shown that microparticulates suspended in water can be collected by continuous centrifugation and examined before and after separation using a sucrose gradient. These fractions have associated with them pesticides of various types, perhaps in a selective way. The usefulness of the standard methods of water analysis for these pesticide compounds was seriously questioned.

Examination of the particulate fractions in Lake Erie has revealed that the most common size particle was in the range of 0.1 μ m. Chlorinated hydrocarbon pesticides such as endrin, aldrin, heptachlor and lindane were found in association with these particles and data suggests that aldrin and heptachlor were found more frequently on the smaller, less dense particles while lindane was associated with the

larger, more dense particulates. Our results have demonstrated that different bacteria in the presence of these compounds could be affected in different ways. The significance of the involvement of such pesticides in the microecology is very great since we know now that microbial cell yields, DNA, RNA, and protein patterns of synthesis can be affected. One survey conducted during these studies showed that of 151 heterotrophic aerobic bacteria isolated from Lake Erie, 55 were stimulated by aldrin, 54 by endrin, and 45 by dieldrin. Forty six cultures were inhibited by aldrin, 43 by endrin and 43 by dieldrin.

Bacteria grown in a concentration of 1.6 mg of dissolved oxygen per liter or in the presence of the chlorinated hydrocarbon pesticide aldrin exhibited a greater cell yield and shorter generation time than cells grown in a medium free of pesticides under control conditions. The cell size, synthetic patterns of DNA, RNA and protein were altered as compared to a control. Increased amounts of poly-beta-hydroxybutyric acid were observed in the stationary phases of growth of bacteria grown in the presence of lowered amounts of oxygen or aldrin. The treated bacteria contained cell wall imperfections termed "pores" and extensive intracytoplasmic membrane systems. All these facts strongly suggest that the presence of chlorinated hydrocarbons in the environment are seriously altering the microbial metabolism in the water and in turn affecting higher life forms in ways we do not yet understand.

Results from experiments on removal of aldrin from lake water using flocculent bacteria were very successful. Floc forming bacteria isolated from Lake Erie adsorbed and concentrated aldrin from a colloidal dispersion so that the settling of the bacterial flocs removed the

chemical from the water phase. Contemporary sediments forming in the lake contain aldrin and could absorb more. These facts will be useful in learning about possible methods of lake purification.

LIST OF THESES AND PRESENTATIONS ATTRIBUTABLE
TO GRANT # A 006 OHIO

Theses

- (1) A study of the effects of three chlorinated hydrocarbon pesticides on the growth of a heterotrophic microbial population isolated from Lake Erie. Tatia A. McNair, M. S.
- (2) A study of Microcystis aeruginosa and its relationship with associated bacteria. Alan J. Johnson, M. S.
- (3) Studies on heterotrophic microorganisms isolated from Lake Erie and Antarctica with emphasis on temperature profiles and DNA composition. Robert Z. Maigetter, M. S.
- (4) Adsorption of the chlorinated hydrocarbon pesticide aldrin by microbial floc and lake sediment and its ecological implications. Walter O. Leshniowsky, M. S.
- (5) Cytological and physiological effects of chlorinated hydrocarbon pesticides and dissolved oxygen concentrations on a Pseudomonas sp. isolated from Lake Erie. Robert Kennedy, Ph.D. (Dissertation)

Presentations

- (1) A numerical analysis of heterotrophic bacteria isolated from Lake Erie and their growth response to chlorinated pesticides, by John M. Gnau, Robert M. Pfister, and Chester I. Randles. Regional meeting of the American Society for Microbiology. French Lick, Indiana.
- (2) Lake Erie microparticulate, pesticide and microorganism interactions, by Robert M. Pfister, J. I. Frea, P. R. Dugan, K. Zaebst, J. A. Duchene, R. Kennedy, and T. McNair. 1970 Abstracts, 13th Conference on Great Lakes Research, p. 38.
- (3) Accumulation of chlorinated hydrocarbons by microbial floc and its ecological implication, by W. Leshniowsky, P. R. Dugan, R. M. Pfister, and J. I. Frea. 1970 Abstracts 13th Conference on Great Lakes Research, p. 37.

- (4) Biological nitrogen fixation potential in Lake Erie, 1970.
D. L. Howard, J. I. Frea, R. M. Pfister and P. R. Dugan. Abstracts
13th Conference on Great Lakes Research, p. 14.
- (5) Effects of chlorinated hydrocarbons and dissolved oxygen on the
metabolic activities on a Pseudomonas species isolated from Lake
Erie, R. S. Kennedy and R. M. Pfister. 1970 Bacteriol. Proc.,
p. 18.
- (6) The isolation characterization of Chromobacterium sp. isolated
from Lake Erie and Antarctica, Robert Z. Maigetter and Robert M.
Pfister. 1970 Regional meeting of American Society for Microbiology,
Battelle Memorial Institute, Columbus, Ohio.

PART A

INFLUENCE OF ALDRIN AND VARIED OXYGEN
CONCENTRATIONS ON A LAKE ERIE HETEROTROPHIC BACTERIUM:
ELECTRON MICROSCOPIC EXAMINATION

R. S. KENNEDY AND R. M. PFISTER
UNIVERSITY OF GEORGIA AND THE OHIO STATE UNIVERSITY

Abstract

Cells grown in a concentration of 1.6 mg dissolved oxygen per liter or in the presence of the chlorinated hydrocarbon pesticide, hexachloro-hexahydro-endo-exo-dimethano-naphthalene (aldrin) exhibited a more rugose cell wall, more polyphosphate as seen with the electron microscope, and more poly-beta hydroxybutyrate than was observed in control cultures. The pesticide treated cells also contained cell wall imperfections termed "pores". Cell wall "blebs" were more prominent in some phases of growth than in others and the phase of occurrence changed with the cultural conditions. All of the treated cells contained a more extensive intracytoplasmic membrane system than the control cells.

Introduction

In a previous communication we reported that the growth response and synthetic capacity of a Pseudomas sp was altered in the presence of the chlorinated hydrocarbon pesticide aldrin (10). In addition the cells were shown to store larger quantities of poly-beta-hydroxybutyric acid (PHB) either in the compound's presence or as oxygen became more limiting.

The purpose of this paper is to describe the cytologic alterations which the bacteria exhibited in the presence of this pesticide.

There have been numerous investigations on the biochemistry, physiology, and morphology of aquatic microorganisms in recent years. Few studies involved the use of the techniques of electron microscopy especially with bacterial forms such as the Pseudomonads. This is significant since these forms of heterotrophic bacteria are one of the principal types found in the aquatic environment. While some strains of marine pseudomonads have been studied with the electron microscope (7, 1, 3, 14, 2, 20, 21) the effect of different environmental conditions on marine pseudomonad fine structure has been reported sparingly (5, 21).

Alterations in the cell membrane and cell wall have been studied in aquatic organisms when cultured under a variety of environmental conditions (5, 18, 8). The rugose appearance of the cell wall and cell wall "blebs" in gram negative bacteria appear constitutive in some organisms and inducible in others. These structures do not seem to be genera specific (6, 11, 18, 20, 12, 19).

Intracytoplasmic membrane formations have been induced under several altered environmental conditions, e.g., lowered oxygen concentrations, complexity of culture medium, and temperature (4, 11, 17, 22, 21).

The purpose of this paper is to present results concerning the effect of the chlorinated hydrocarbon aldrin and varying dissolved oxygen concentrations on an aquatic (fresh water) pseudomonad.

Materials and Methods

Organism. The organism used in this study was a Pseudomonas sp. isolated from the western basin of Lake Erie. The growth characteristics and conditions have been described in another paper (10). The culture vessel was a "Fermentation Design" 5 liter fermentor equipped to monitor and control agitation, oxygen level, temperature, and pH. Three liters of a medium (AGS) consisting of (per liter distilled H₂O): arginine HCl, 0.5 g; glucose, 5.0 g; MgSO₄ · 7H₂O, 0.2 g; K₂HPO₄, 2.0 g; and KH₂PO₄, 1.0 g, were placed into the fermentor vessel and sterilized. A 3.0 percent inoculum final concentration in the fermentor was employed after the sterile unit had cooled to 20 C and represents the lag phase samples taken during the experiment. Where aldrin was used 1.95 mg of the pure compound were added directly to the sterile fermentor vessel while it was still hot. The initial oxygen concentration was set to 8.9 mg D.O. per liter using the control device. When reduced amounts of O₂ were desired the control unit was reset immediately after inoculation and the respiration of the growing bacteria reduced the O₂ level to either 1.6 or 0.8 mg D. O. per liter.

The optical density of the culture fluid was monitored and recorded continuously and was used as a guide to determine when samples should be taken for electron microscopy (10). Samples were removed during the lag, early log, mid log, late log, and stationary phases of the growth cycle and frozen in liquid N₂ prior to subsequent fixation and embedding.

Electron Microscopy. Cell samples were removed and fixed for 1 hour in 3 percent glutaraldehyde adjusted to pH 7.2 with 0.1 M sodium cacodylate and 0.1M HCl. The fixed cells were washed 5 times in fresh cacodylate buffer (pH 7.2) and post-fixed in 1 percent osmium tetroxide according to the method of Kellenberger, Ryter, and Sechaud (9). The fixed material was dehydrated and embedded in Epon 812 according to Luft (13).

Silver-gray sections were cut with glass or diamond knives on a Reichert OMU2 or LKB Ultratome III ultra microtome. The sections were collected on uncoated 300 mesh copper grids and stained 5-20 minutes in lead citrate (16).

Freeze etching. The cells were frozen-etched by placing a thick suspension on a gold specimen cap, freezing in Freon-22, and then transferring the sample to liquid nitrogen. The frozen specimen was transferred to a Balzers Freeze-etching unit, evaluated and fractured. The specimens were etched 1.5-2.0 min at -100 C, shadowed with platinum, and carbon coated. The replica was floated free in distilled water, cleaned in 70 percent sulfuric acid (30 min), rinsed in distilled water, placed in chlorox (1 hr), and followed by several rinses in distilled water prior to placement on a 300-mesh uncoated copper grid. The specimens were examined in an Hitachi HS-8 or Zeiss EM 95

electron microscope at 50 or 60 Kv accelerating voltages respectively.

Results

Cytological Observations. Cells in the control culture exposed to 8.9 mg D.O. per liter in the lag phase of growth (Fig. 1) were characterized by a few polyphosphate (PP) granules and a few electron transparent granules presumed to be poly-beta-hydroxybutyrate (PHB) (10). The deoxyribonucleic acid (DNA) found in these cells (N) was distributed throughout the central portion of the cell. Several cell wall "blebs" (b) were detected during this phase and can be seen in these sections.

Ultra thin sections of cells grown in the control culture and harvested during the early log phase of growth (Fig. 2) showed a decrease in the PHB content. The nuclear material (N), distributed throughout the central portion of the cell, cell wall "blebs", and "blebs" free in the medium were observed during this phase of growth. The cell wall (W) was the typical multi-layered structure seen in Gram negative microorganisms and did contain occasional mesosomes (M) or a limited internal membrane system. Polyphosphate granules observed in the lag phase were not observed in the early log phase of growth.

Control cells harvested during the mid log phase (Fig. 3), had a slightly rugose cell wall (W) containing few evaginations or "blebs" (b) (Fig. 4).

The late log phase of growth (Fig. 5) of the control culture is characterized by more abundant internal membrane (M) formation within the cells and a slightly rugose cell wall (W).

HIB or polyphosphate granules were not seen to any extent during the stationary phase of growth (Fig. 6). Internal membranes (M) were observed in these cells (Fig. 7) and the cell wall (W) appeared normal or slightly rugose. The periphery of the cytoplasm was filled with ribosomal (R) material and occasional cell wall "blebs" (b) were observed.

Bacteria harvested and sectioned during the lag phase of growth exposure to 0.65 mg aldrin per liter and 8.9 mg D. O. per liter were structurally the same as the control cells. This was also true for cells removed from the lag phase of growth when they were cultured under 1.6 mg D. O. per liter and 0.8 mg D. O. per liter. For this reason, electron micrographs of this phase exposed to these different environmental conditions have not been included.

Thin sections of cells cultured in the presence of 0.65 mg aldrin per liter and removed from the fermentor during the early log phase of growth (Fig. 8) showed no more external vesicular organelles or cell wall "blebs" than did previous control specimens. The nuclear material (N) of these cells was abundant and concentrated in the central portion of the cells. Numerous large mesosomes (M) were observed in the cells and the cell wall (W) appeared as a rugose surface.

Cells cultured under 0.65 mg aldrin per liter and harvested in the mid log phase of growth (Fig. 9) showed an even distribution of nuclear material (N) within the cells. Cell wall "blebs" (b) were observed on the surface of the bacteria as well as free in the surrounding space adjacent to the cells. The cell wall (W) appeared to be more rugose when compared to cells taken from the same time period of the control culture. Numerous pores (p), in the cell wall were observed

at this stage of the growth cycle. These pores were distributed over the surface of the bacteria in a reasonably uniform manner. The arrows in Figure 9 point to a number of these openings in the wall and careful examination of the rest of the cell will show even more of these structures. An enlargement of these pores (p) (Fig. 10) reveals that they appear as complete separations in the lipopolysaccharide-protein outer envelop. The intact cytoplasmic membrane (CM) and peptidoglycan (MU) layer can be seen underneath the pores (p) which appear as an opening of about 2-4 nm.

Bacteria harvested from the late log phase of growth of cultures grown in the presence of aldrin (fig. 11) showed an increase in presence of cell wall "blebs" (b) and had a more complex internal membrane system (M). The cell wall pores (p) observed during the mid log phase of growth were also present in these specimens.

During the stationary growth phase of cells grown in the presence of aldrin, many PHB granules (Figures 12, 13) were observed. The presence of PHB in these bacteria had been shown chemically in another paper (10). Granules were not readily observed in the stationary phase of the control culture. The cell walls (W) of nearly all cells examined were very rugose and the internal membranes (M) of the cells (Figures 12, 13) appeared more extensive. Cell wall "blebs" (b) were observed in all the thin section preparations. A freeze-etching preparation was examined (Figure 14) and found to possess a rough surface structure (RS) along the outer wall (OW).

Cells harvested during the early log phase of growth (Fig. 15) in cultures which had been prepared and grown under a reduced concentration of oxygen (1.6 mg D. O. per liter) contained some mesosomes (M) or internal membranes and a limited number of cell wall "blebs" (b). The cell wall and cytoplasmic membrane (CM) of the organisms grown under these conditions were fairly uniform and no unusual features were noted.

Cells harvested during the mid log phase of growth (Fig. 16) in cultures that had been grown under 1.6 mg D. O. per liter were found to contain wall "blebs" (b) which became more prominent during this time period than during the early log phase of growth. A few PHB granules were observed but were not a characteristic feature during this phase of growth and the cell wall of some of the organisms was rugose in appearance. The cytoplasmic membrane (CM), cell wall (CW), and ribosomes are clearly evident in this specimen.

Cells taken from the late log and stationary phases of growth of cultures grown under 1.6 mg D. O. per liter revealed many PHB granules and numerous polyphosphate (PP) inclusion bodies (figures 17, 18). Figure 17 shows a freeze-etch preparation of one of the stationary phase cells revealing numerous PHB granules in the cytoplasm (CC). The stretching of the elastic-like PHB polymer can be seen in a number of the internal granules. Thin sections of these bacteria (Figure 18) reveal numerous PHB bodies in the cytoplasm, polyphosphate granules (PP), a normal cytoplasmic membrane (CM), and slightly rugose cell wall (W). Many of the organisms were shown to contain a more complex inner membrane system (IM) (Fig. 19). A summary of the results found during the study is shown in Table 1.

Discussion

A number of ultrastructural differences in the bacteria grown in the presence of aldrin or reduced amounts of dissolved oxygen were apparent during these studies. The cells were found to have an increased occurrence of "blebs" located on the outer surface of the cell wall. These "blebs" are believed to originate in the wall itself and appear to be released into the surrounding medium. Close examination of these "blebs" (Fig. 4) suggests that they are a bilayered structure resulting from a pinching off of the outer envelope.

The function of the cell wall "blebs" is not known but they have been observed in other organisms (21, 12). The "blebs" might have been formed to increase the surface area of the bacteria in order to enable a more efficient transfer of nutrients and/or waste products.

The rugose appearance of the cell wall observed in the pesticide treated cells may be the result of the pesticide interfering with the synthesis of cell wall or it may be that lipid-soluble pesticides are altering the structure of the wall. The alteration could be due to the interference of hydrocarbons in energy yielding or producing processes with the rugose appearing wall occurring as secondary result. The fact that the metabolism of these bacteria was altered and that they have been shown to store PHB under conditions of either low oxygen or the presence of pesticide (10) suggests that these chemicals are interfering with some basic components of the cell's living system.

The pores observed in bacteria treated with aldrin are interesting especially with respect to the permeability of the cells to either nutrients or the pesticides themselves. These holes in the lipopoly-saccharide-protein wall may be the result of a lesion caused by the release of the cell wall "blebs" into the surrounding medium. In other words, the cell wall itself appears to be in an increased dynamic state when in contact with the hydrocarbon aldrin. The pores may be sites where the interaction of lipophilic pesticides and lipid containing wall occurs. The results (Figure 9) suggest that the pores are rather evenly spaced and may represent sites where the pesticide, which is not soluble in the medium and acts as a particle, has interacted with the wall and caused local damage. This concept seems difficult to accept because it implies that the wall has sustained damage and could have negative effects on the growth of the organisms. This does not appear to be true and in this case the growth of the culture was markedly stimulated (10).

There does appear to be an increase in the content of internal membranes in cells which have been grown in lowered amounts of oxygen or in the presence of pesticides. The increased membrane content may increase the efficiency of utilization of available oxygen, assuming the membrane is the site of respiratory enzyme activity. An inhibition or feedback mechanism in the regulation of membrane formation by the lowered available dissolved oxygen concentration might result in the increased membrane content.

A strong interrelationship is suggested between chlorinated pesticide presence and dissolved oxygen concentrations. The effects of reducing the oxygen concentration or adding pesticide to the culture medium are remarkably similar. There are some differences in synthetic capacities of the bacteria (10), but the total effect of these alterations on the cytology of the cells is similar. We have concluded that there is evidence that the chlorinated hydrocarbon pesticides do interfere with metabolic processes in bacteria in some way and that this involvement is reflected in a manner similar to the response of these bacteria to reduced concentrations of available oxygen.

The culture used during this study was recently isolated from Lake Erie and the pesticide aldrin is also known to exist in this body of water. Since many of the heterotrophic bacteria in Lake Erie are known to respond in some way to these compounds (15), it is assumed that similar metabolic effects will be observed in the Lake. The increase in growth of these bacteria in the presence of such recalcitrant molecules is important in the total carbon balance of a body of water and aids in speeding the eutrophication process in Lake Erie.

Table 1 Ultrastructural differences observed in cells grown under different environmental conditions

Culture Condition	Phase of Growth				
	Lag Phase	Early Log	Mid Log	Late Log	Stationary
Control	Poly PO_4 Some PHB	No poly PO_4 No PHB	Few "blebs"	Few "blebs" IM	Few "blebs" Low PHB No poly PO_4 Smooth CW ⁴
0.65 mg Aldrin/liter 8.9 mg D.O./liter	Poly PO_4 Some PHB	IM Cell Wall Rugose	Some "blebs" Cell Wall Rugose Pores in wall	IM Cell Wall Rugose Many pores Some PHB	Numer PHB Some poly PO_4 Some "blebs" Cell Wall Rugose IM
1.6 mg D.O./liter	Poly PO_4 Some PHB	IM Cell Wall Smooth Few "blebs"	IM Slightly Rugose Some PHB	IM Some Walls Rugose Some PHB Poly PO_4	IM Slightly Rugose Numer. PHB Some Poly PO_4 Some "blebs" ⁴
0.8 mg D.O./liter	Poly PO_4 Some PHB	IM Slightly Rugose Some "blebs"	IM Walls Rugose More "blebs"	IM Walls More Rugose Some PHB	IM Wall Rugose on some Many "blebs" Numer PHB

PHB = poly-beta-hydroxybutyrate

IM = internal membranes

CW = cell wall

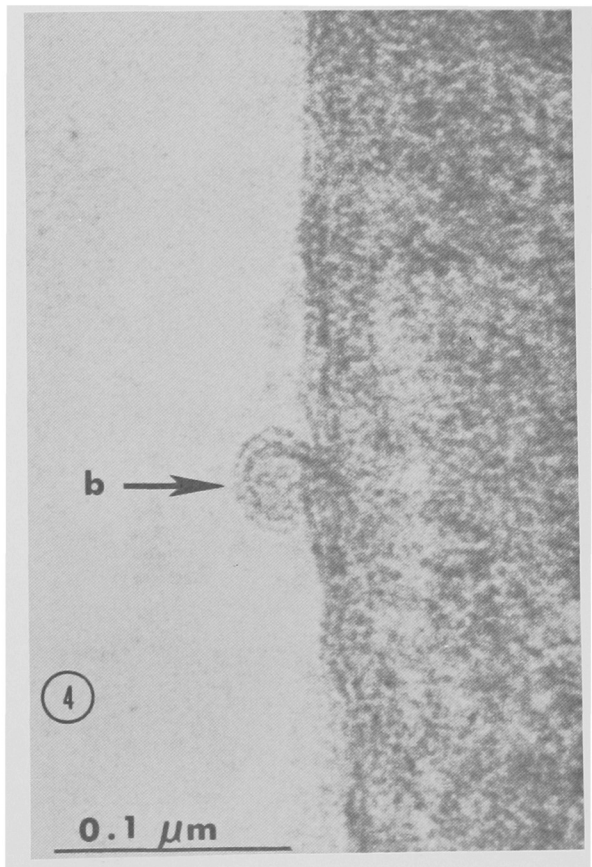
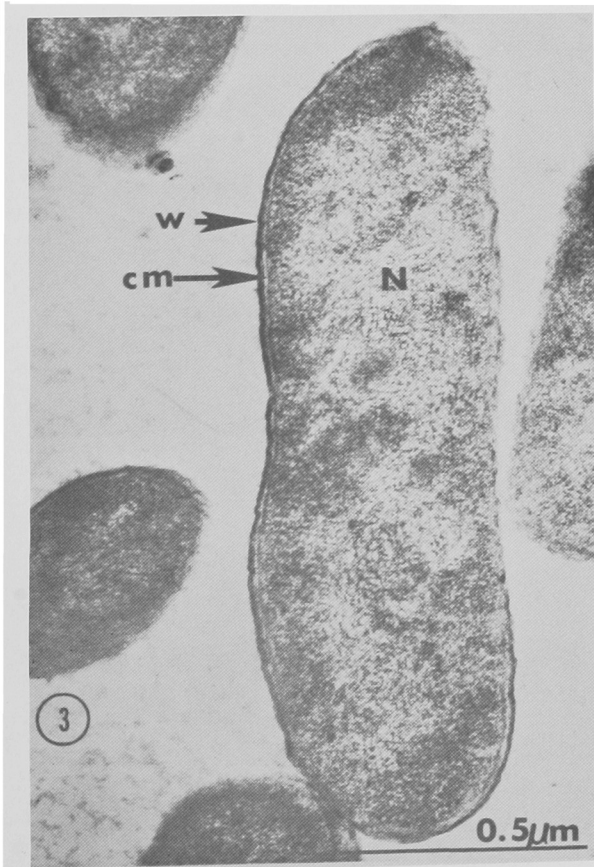
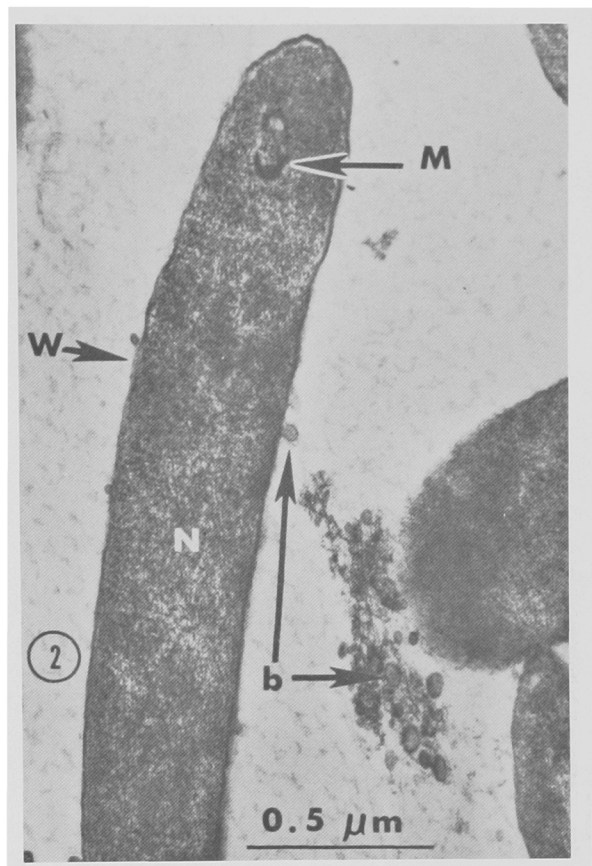
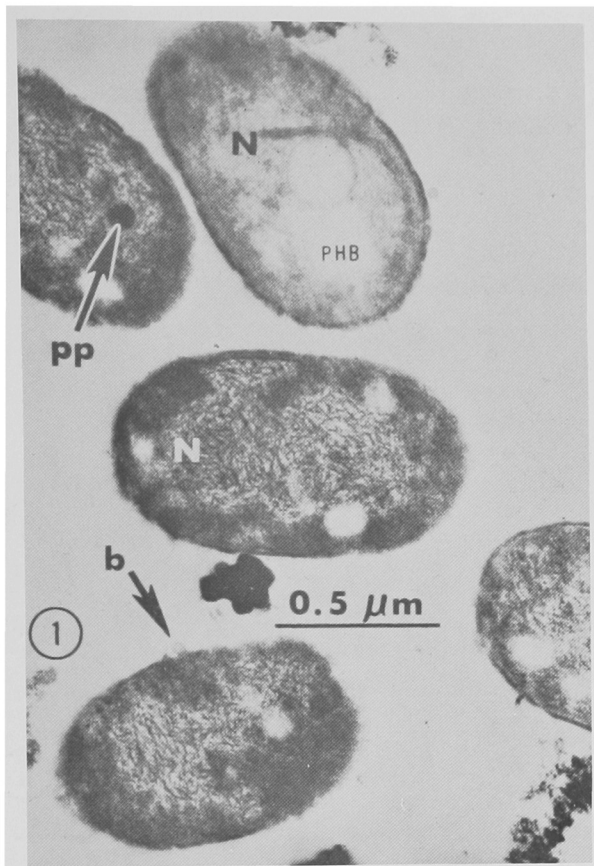
Poly PO_4 = polyphosphate

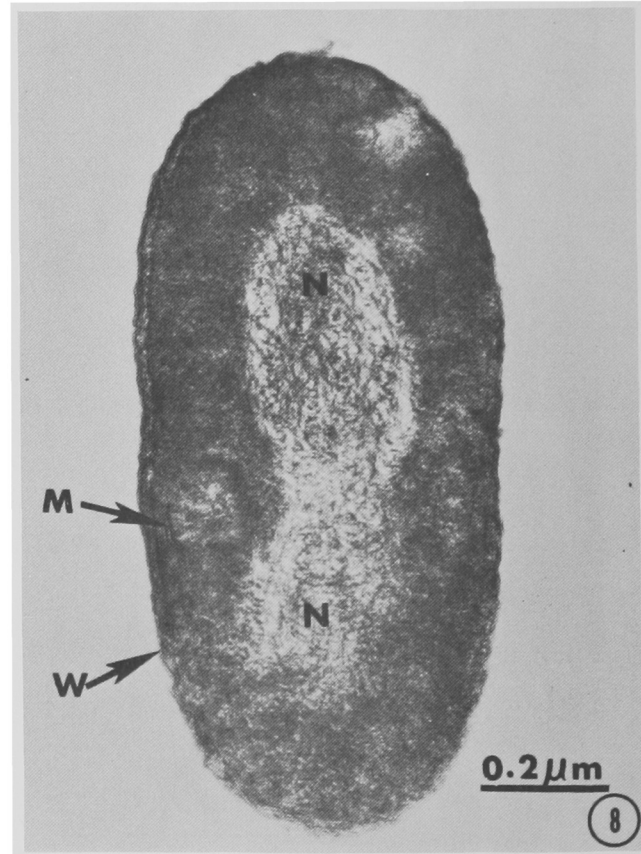
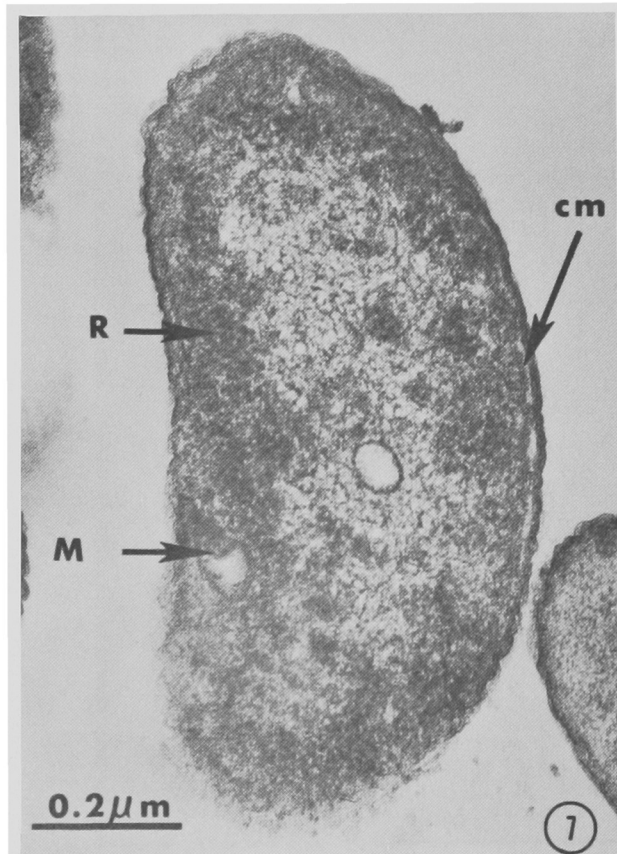
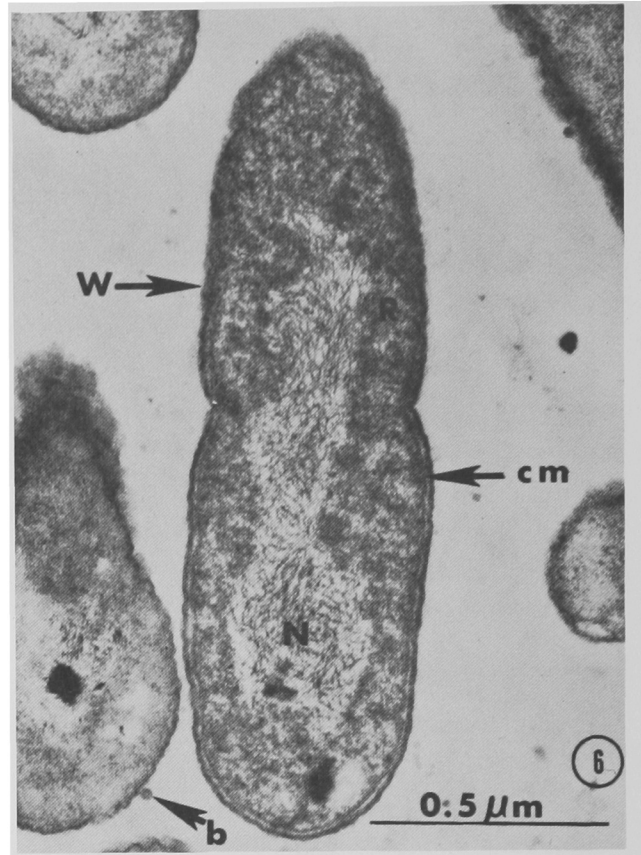
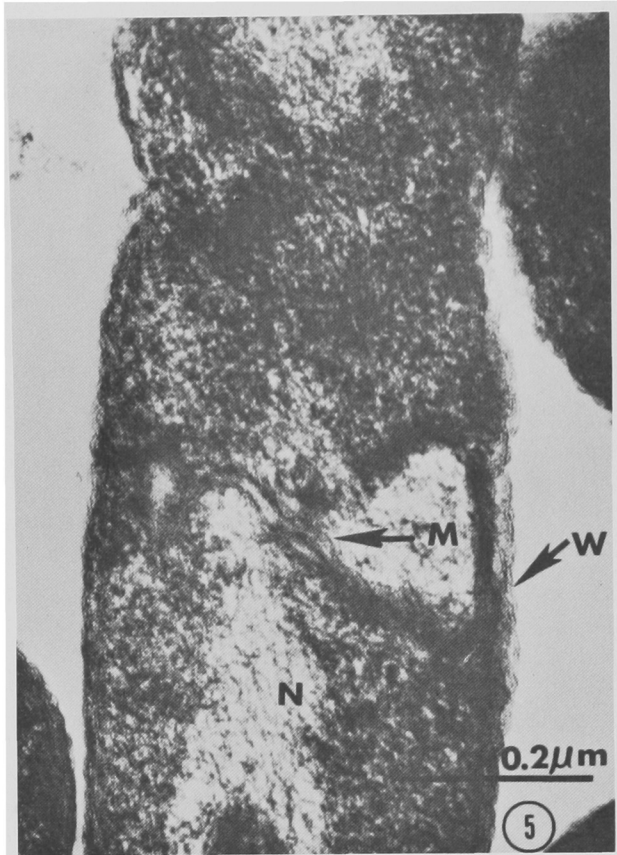
Numer PHB = numerous PHB granules

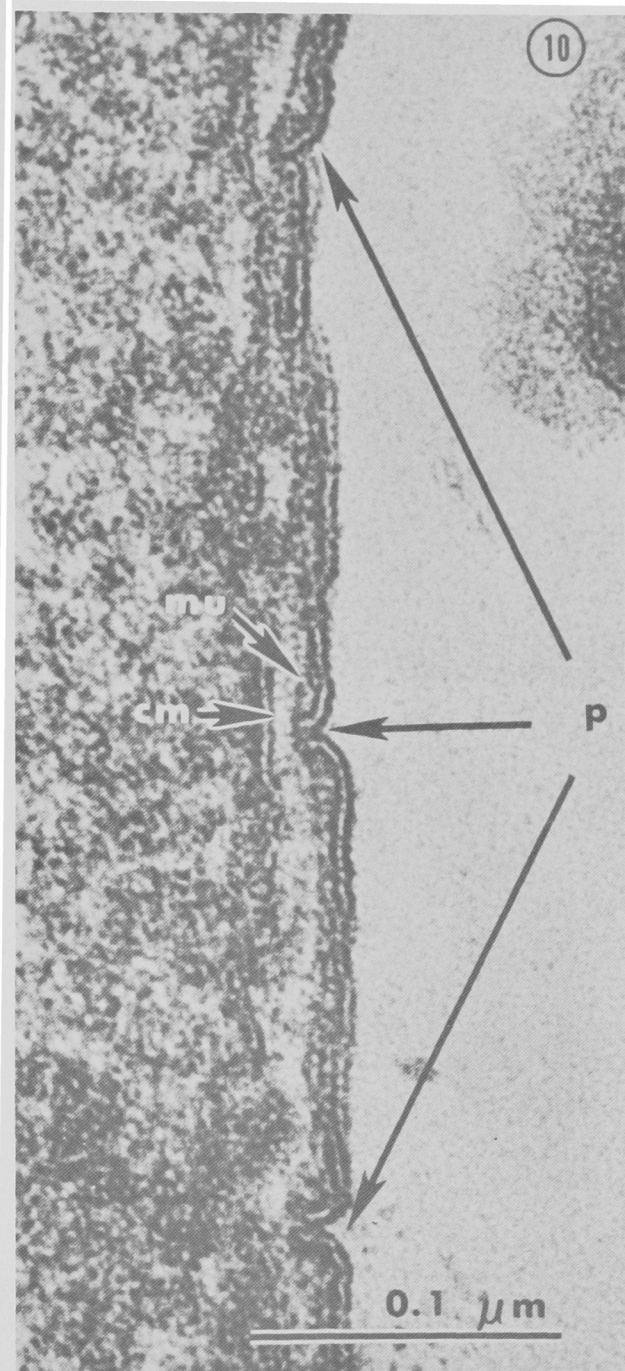
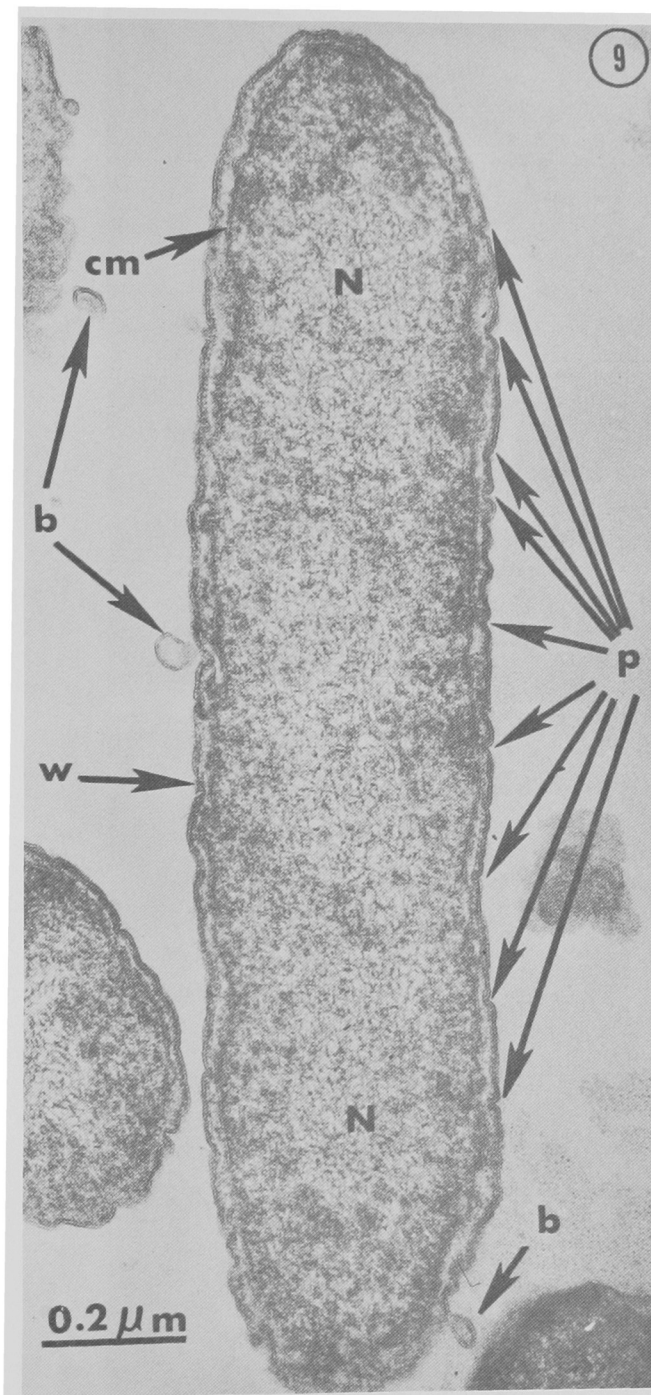
Literature Cited

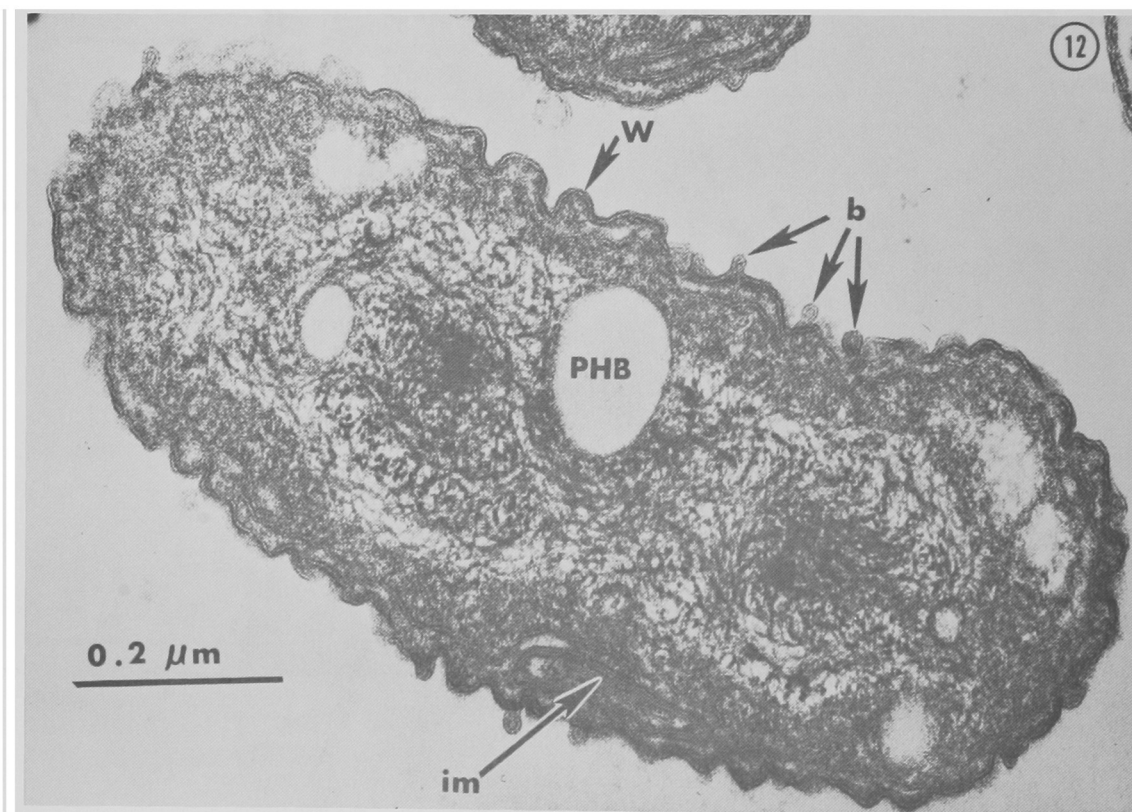
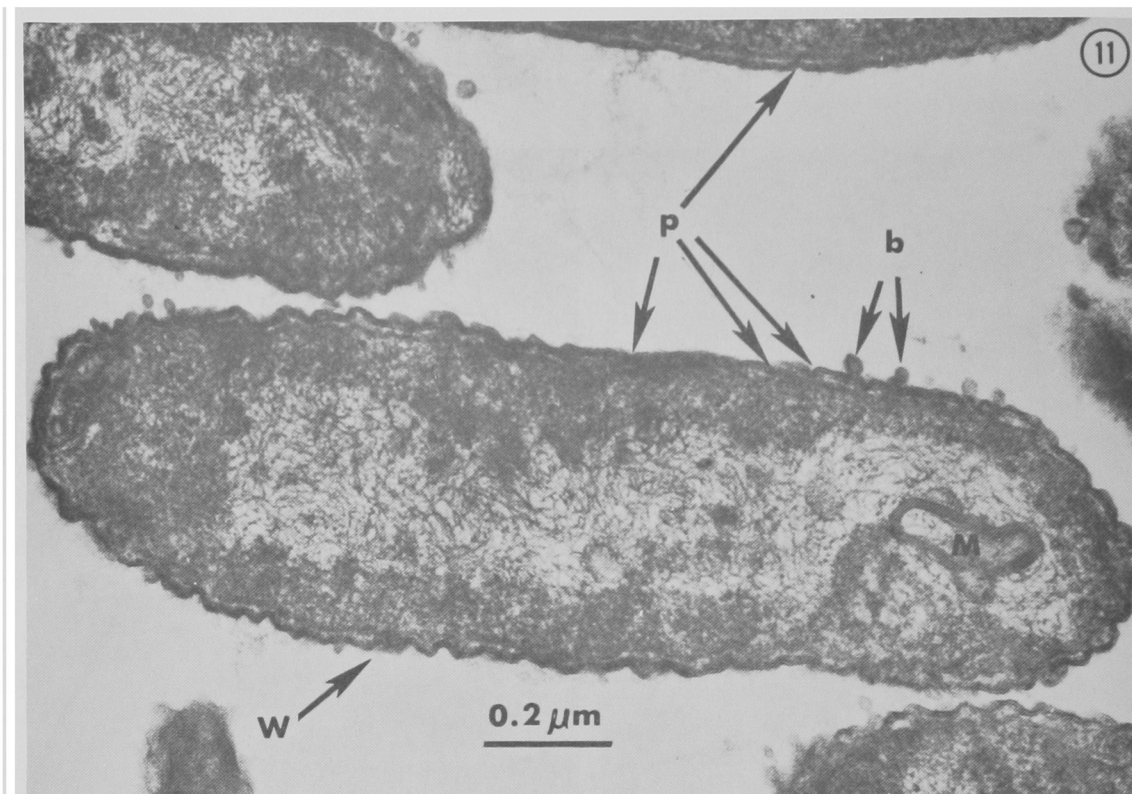
1. Brown, A. D. 1961. Effect of salt concentration during growth on properties of the cell envelope of a marine pseudomonad. *Biochem. Biophys. Acta*, 49: 585-588.
2. Brown, A. D. 1964. Aspects of bacterial response to the ionic environment. *Bacteriol. Rev.*, 28: 296-329.
3. Brown, A. D., D. G. Drummond, and R. J. Node. 1962. The peripheral structures of gram-negative bacteria. II. Membranes of bacilli and spheroplasts of a marine pseudomonad. *Biochem. Biophys. Acta*, 58: 514-531.
4. Cohen-Bazier, G., R. Kunisawa, and J. S. Poindexter. 1966. The internal membranes of Caulobacter crescentus. *J. Gen. Microbiol.* 42: 301-308.
5. Costerton, J. W., C. Forsberg, T. Matula, F. L. A. Buckmire, and R. A. MacLeod. 1967. Nutrition and metabolism of marine bacteria. XVI. Formation of protoplasts, spheroplasts, and related forms from a Gram-negative marine bacterium. *J. Bacteriol.*, 94: 1764-1777.
6. De Petris, S. 1965. Ultrastructure of the cell wall of Escherichia coli. *J. Ultrastructure Res.*, 12: 247-259.
7. Houwink, A. L. 1956. Flagella, gas vacuoles, and cell wall structure in Halobacterium halobium; an electron microscope study. *J. Gen. Microbiol.*, 15: 146-150.
8. Keeler, R. F., A. E. Ritchie, J. H. Bryner, and J. Elmore. 1966. The preparation and characterization of cell walls and the preparation of flagella of Vibrio fetus. *J. Gen. Microbiol.*, 43: 439-454.
9. Kellenberger, E., A. Ryter, and S. Sechaud. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phase DNA as compared with normal bacterial nucleoids in different physiological states. *J. Biophys. Biochem. Cytol.* 4: 671-676.
10. Kennedy, R. S., and R. M. Pfister, 1970. Influence of Aldrin and varied Oxygen concentrations on a Lake Erie heterotrophic bacterium: Growth characteristics and Biosynthetic patterns.
11. Knox, E. W., M. Vesik, and E. Work. 1966. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of Escherichia coli. *J. Bacteriol.*, 92: 1206-1217.
12. Koike, M., K. Iida, and T. Matsuo. 1969. Electron microscopic studies on mode of action of polymyxin. *J. Bacteriol.*, 97: 448-452.

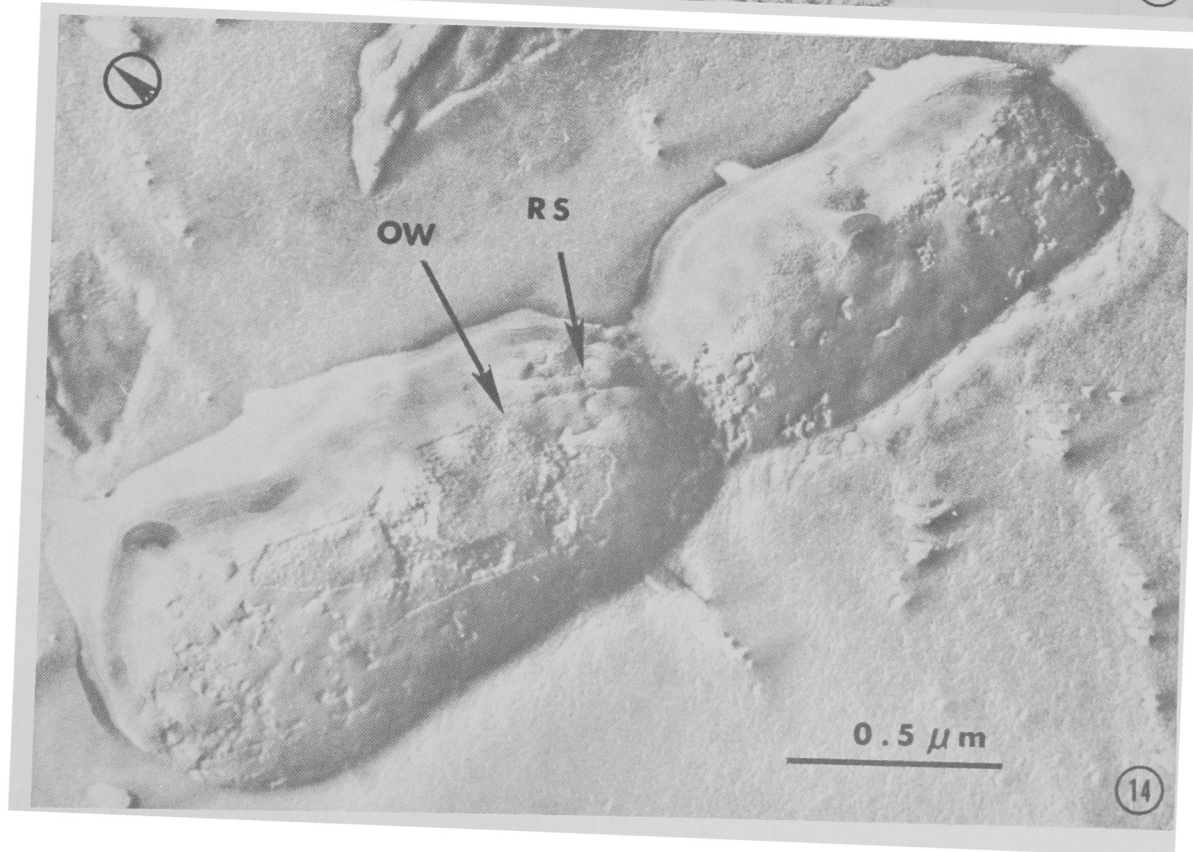
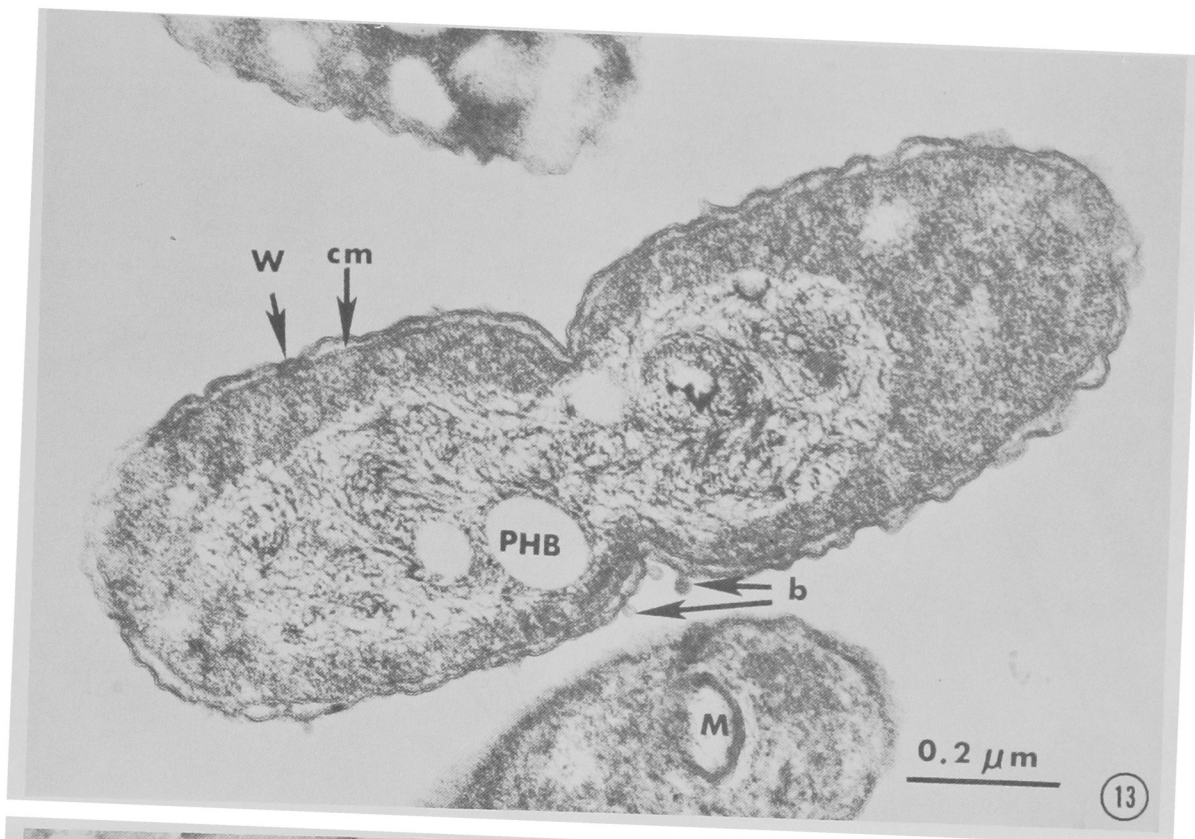
13. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9: 409-414.
14. Murray, R. G. E. 1963. On the cell wall structure of Spirillum serpens. *Can. J. Microbiol.*, 9: 381-392.
15. Pfister, R. M., J. I. Frea, P. R. Dugan, C. I. Randles, K. Zaebst, J. Duchene, T. McNair, and R. Kennedy. 1970. Chlorinated hydrocarbon, microparticulate effects on microorganisms isolated from Lake Erie. *Proc. 13th Conf. Great Lakes Res.*: 82-92.
16. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17: 208-212.
17. Schnaitman, C. and J. W. Greenawalt. 1966. Intracytoplasmic membranes in Escherichia coli. *J. Bacteriol.*, 92: 780-783.
18. Shaw, M. K. 1968. Formation of filaments and synthesis of macromolecules at temperatures below the minimum for growth of Escherichia coli. *J. Bacteriol.*, 95: 221-230.
19. Shively, J. W., G. L. Decker, and J. W. Greenawalt. 1970. Comparative ultrastructure of the thiobacilli. *J. Bacteriol.*, 101: 618-627.
20. Wiebe, W. J. and G. B. Chapman. 1968a. Fine structure of selected marine pseudomonads and achromobacters. *J. Bacteriol.*, 95: 1862-1873.
21. Wiebe, W. J. 1968b. Variation in the fine structure of a marine Achromobacter and a marine pseudomonad grown under selected nutritional and temperature regimes. *J. Bacteriol.*, 95: 1874-1886.
22. Wiegand, R. A., J. M. Shively and J. W. Greenawalt. 1970. Formation and ultrastructure of extra membranes in Escherichia coli. *J. Bacteriol.* 102: 240-249.

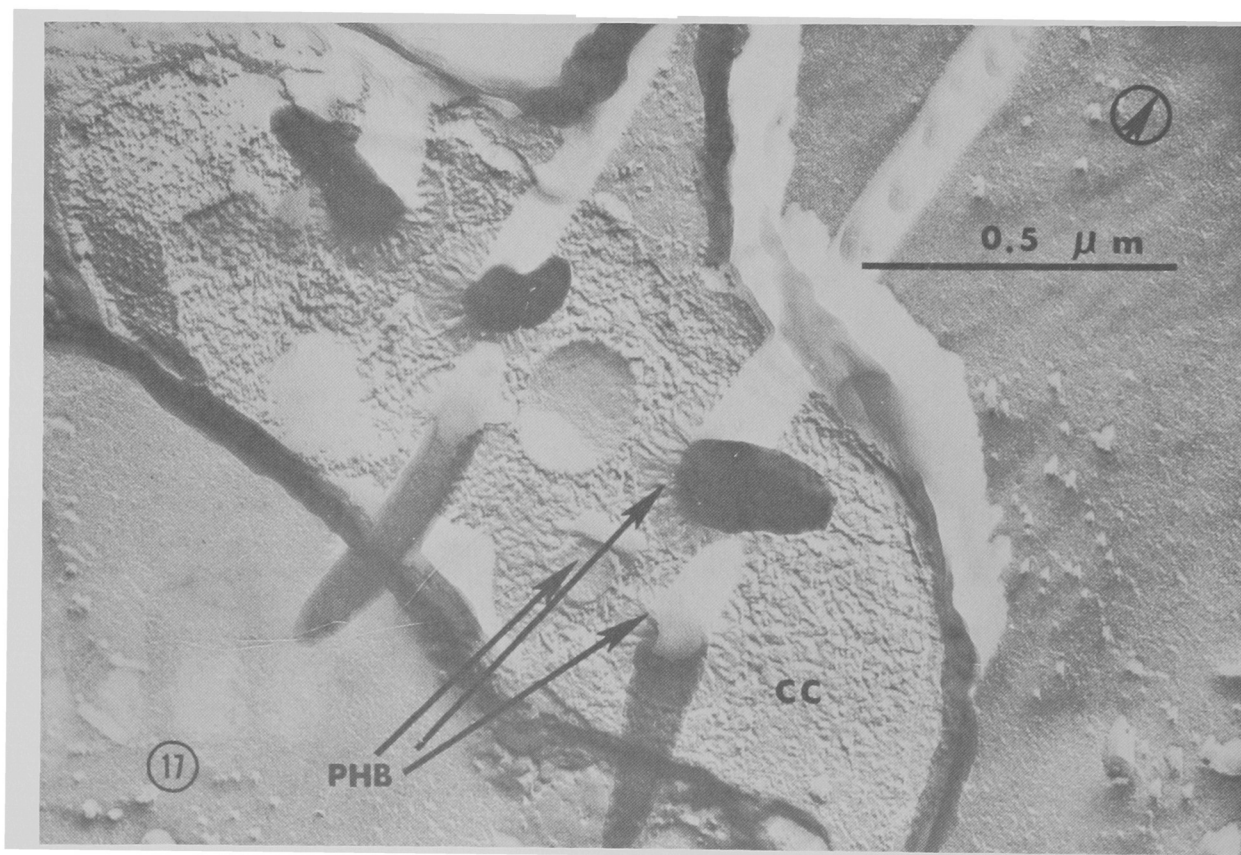
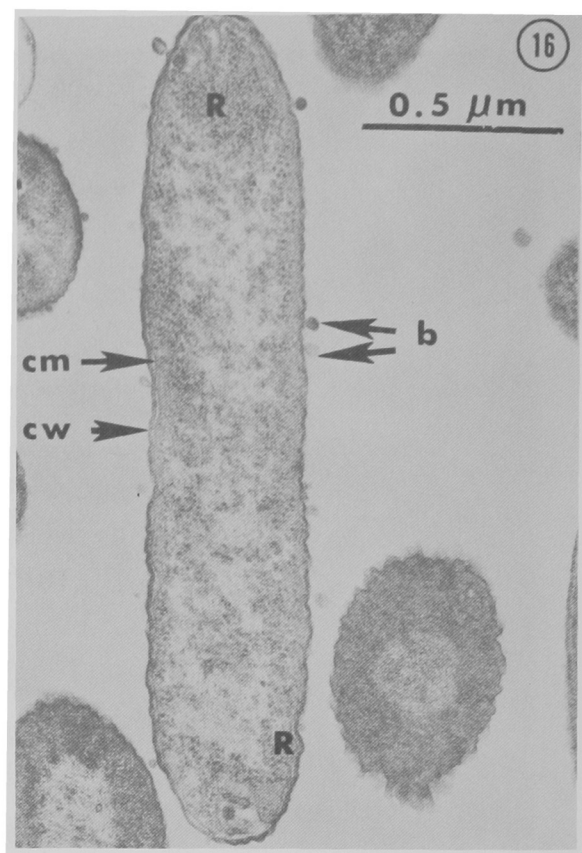
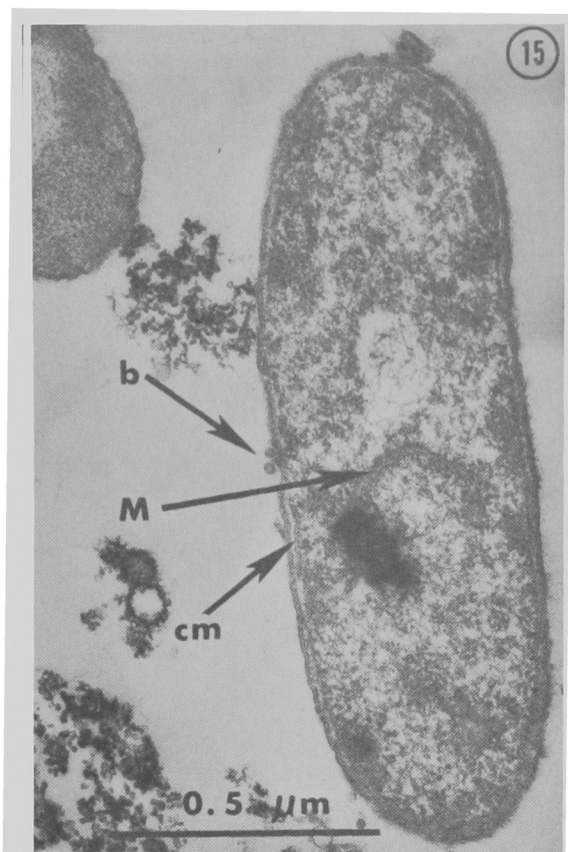


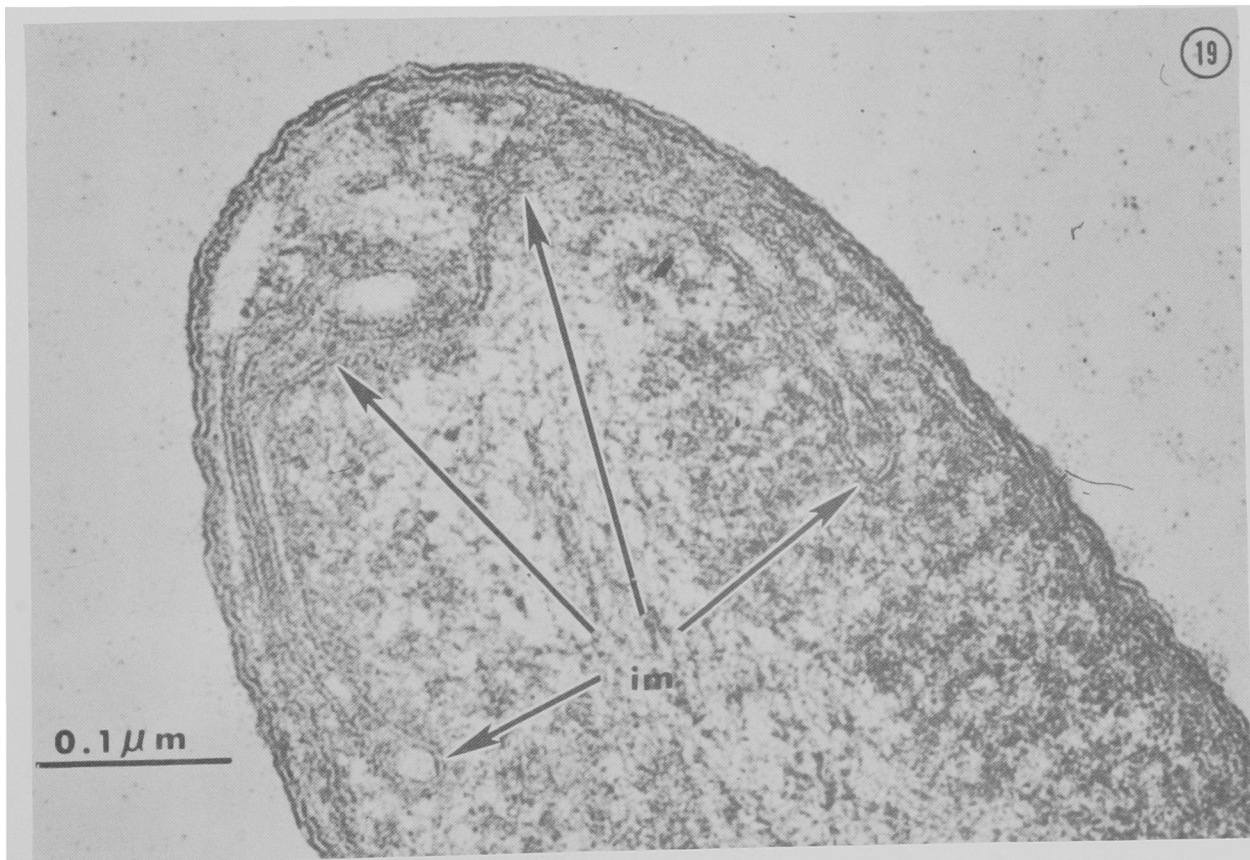
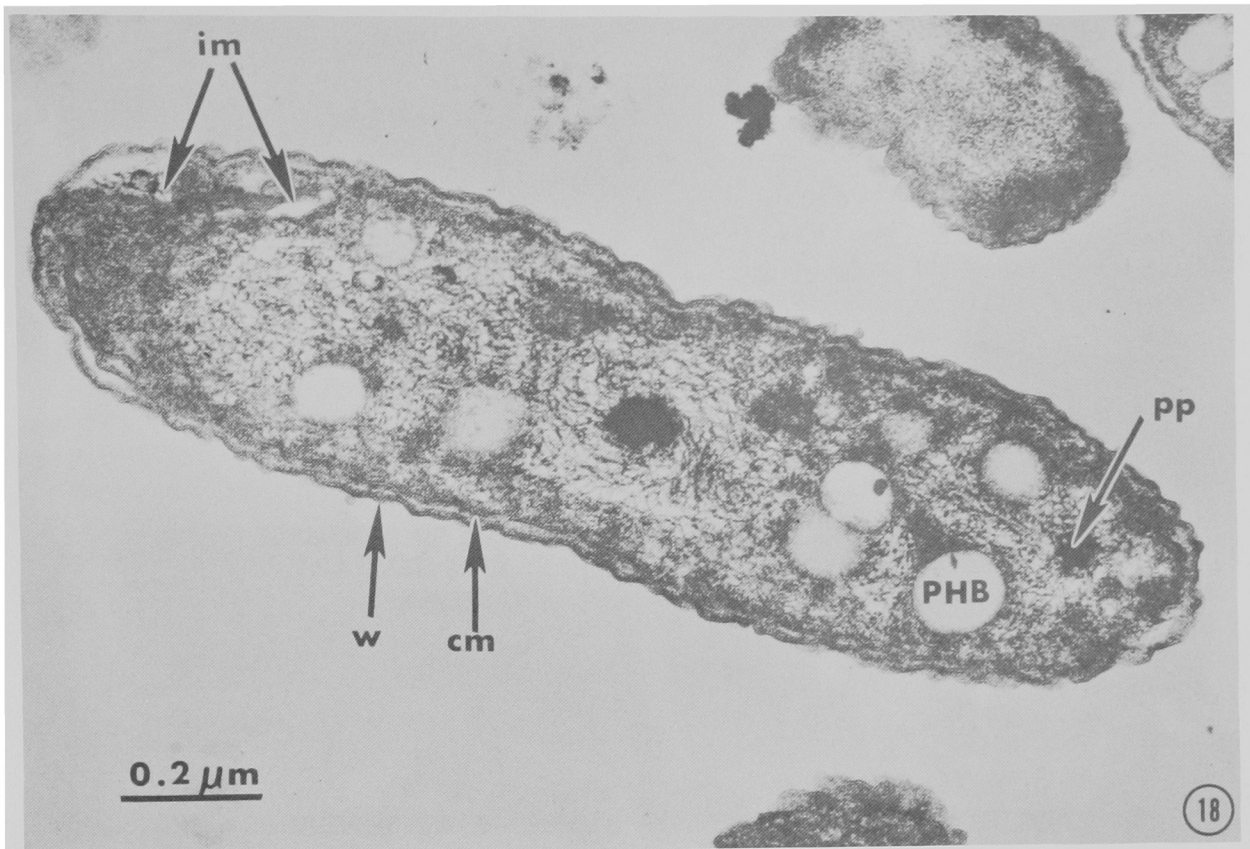












PART B

INFLUENCE OF ALDRIN AND VARIED OXYGEN CONCENTRATIONS ON
A LAKE ERIE HETEROTROPHIC BACTERIUM: GROWTH
CHARACTERISTICS AND BIOSYNTHETIC PATTERNS

R. S. KENNEDY AND R. M. PFISTER
UNIVERSITY OF GEORGIA AND THE OHIO STATE UNIVERSITY

Abstract

A Pseudomonas sp isolated from Lake Erie and grown in batch culture in the presence of the chlorinated hydrocarbon pesticide aldrin or under reduced (below saturation) dissolved oxygen concentrations had a greater cell yield and shorter generation time than cells grown in a medium free of pesticides under control conditions. The cell size of the organisms was reduced when cultured in the presence of chlorinated hydrocarbon pesticides or a reduced dissolved oxygen content. The synthetic patterns of DNA, RNA, and protein were altered when compared to the control cells. The pattern of carbohydrate synthesis was not significantly changed in the presence of pesticides or a reduced dissolved oxygen concentration. Increased amounts of poly-beta-hydroxybutyrate were observed in the stationary phases of growth of bacteria grown in the presence of lowered amounts of dissolved oxygen or in the presence of 0.65 mg aldrin per liter when compared to control cells.

Introduction

The refractory compounds called chlorinated hydrocarbon cyclodienes or more commonly chlorinated pesticides have become of intense interest to the scientific community. Chlorinated hydrocarbon pesticides in the soil can have a definite effect of the growth of microorganisms as well as other organisms constituting the normal flora. These effects range in quality from inhibition to stimulation of growth.

Bacterial cell counts (7), as well as fungal growth (6), were shown to have been affected by the presence of hexachlorohexahydroendo-exo-dimethano-naphthalene (aldrin) in the soil. Dichloro diphenyl trichloro-ethane (DDT) and 1,1-dichloro-2,2 bis (p-chlorophenyl) ethane (DDT) in the soil have also been implicated in a shift in the species composition in the soil (10).

High oxygen tensions inhibit the growth of many aerobic and facultatively anaerobic bacteria (15). Efficient aeration of bacterial cultures has been shown to reduce the number of organisms present in the stationary phase, (18, 23) or inhibit initiation of growth (4). The exact mechanism of oxygen toxicity is not known but Gottlieb (1965) has reviewed some of the theories.

Lake Erie is a body of water containing a variety of chlorinated hydrocarbon pesticides (21) and a fluctuating dissolved oxygen concentration. This study was undertaken to establish the effects of the chlorinated hydrocarbon aldrin and varying dissolved oxygen concentrations on the growth of a pseudomonad isolated from Lake Erie.

Materials and Methods

The culture system. The microorganism used throughout this investigation was a motile Pseudomonas sp isolated from the western basin of Lake Erie in November of 1967 (20). The organism was maintained in a defined (AGS) medium consisting of (per liter double distilled H₂O): Arginine HCl, 0.5g; Dextrose, 5.0g; MgSO₄·7H₂O, 0.2g; K₂HPO₄, 2.0g; and KH₂PO₄, 1.0g.

The culture vessel was a commercially available (Fermentation Design) 5-liter fermentor equipped to monitor and control pH, temperature, and dissolved oxygen. An actively growing culture (30 ml) was used to inoculate 3 liters of medium. All cultures were grown and maintained at pH 8.0 by addition of 2N NaOH or 2N HCl. Growth was monitored by continuously measuring the absorbancy of the culture of 610 nm in a spectrophotometer. The sample was pumped from the fermentor jar, through a flow-through spectrophotometer cuvette and back to the culture vessel. The resulting curves were plotted using a recorder attached to the spectrophotometer.

The appropriate amount of pesticide was added directly to the vessel after autoclaving while the medium was hot. Inoculation was carried out after the vessel had cooled to 20 C using 30 ml of an actively growing culture.

The initial oxygen concentration in the sterile medium was set at 8.9 mg dissolved oxygen (D.O.)/l using the dissolved oxygen control device. During experiments where 8.9 mg D.O./l was required throughout the incubation time the equipment was left at the appropriate setting.

During experiments where reduced amounts of oxygen in the medium were desired the D.O. monitoring and controlling unit was reset at the time of inoculation to the lowered quantity which was either 1.6 or 0.8 mg D. O./l. The impeller of the fermentor was set to fluctuate between 50-200 RPM depending upon which of the dual air flow metering systems was being used (high or low) to adjust the oxygen concentration. The initial depletion of oxygen from 8.9 to either 1.6 or 0.8 mg D. O./l occurred through the respiration of the bacteria and when the O_2 level reached the desired end point, the control unit automatically held it there.

Incubation of the cells was at 20 C for the duration of any experiment. Samples were removed from the culture vessel during the lag, early log, mid log, late log, and stationary phases of growth and either immediately frozen in liquid N_2 or subjected to further testing.

Bacterial size. The cell sizes of bacteria were determined by placing a small drop of cell suspension on a glass microscope slide coated with a thin film of "Difco" Plate Count Agar (PCA). The cell suspension was covered with a cover slip and phase contrast photographs taken on a Zeiss "Photomicroscope" using Panatomic-x film (Kodak). Actual cell size analysis was made by examining the photographs on a Zeiss-Endter "TGZ-3 Particle Size Analyzer." The particle size analyzer was operated on the linear class interval range, standard measuring range, and distribution curve (E). The student T test was used to determine the significance of differences between the mean size value of the population taken from different portions of the growth curve.

Total cell numbers were estimated by a Petroff-Hauser counting chamber. Viable cell numbers were estimated by the spread plate method using PCA. Samples were removed from the fermentor, diluted in sterile water blanks and a 1.0 ml aliquot was plated on pre-cooled agar (PCA) Petri plates. Colonies were counted after 24 hr incubation at 20 C. All samples were plated in triplicate.

Duplicate samples were removed, centrifuged, washed twice with distilled water and resuspended in distilled water for dry weight determinations. A 1 ml aliquot of cell suspension was placed in pre-dried aluminum weighing pans and dried at 100 C to a constant weight.

Biochemical determinations. A 50 ml aliquot of frozen cells was thawed, centrifuged 5,000 x g for 15 min., washed 3 times in distilled water and subjected to ultrasonic treatment using maximum power of a "Bronwill" Biosonik III ultrasonic generator for 3 min. The sonicated suspension was centrifuged at 4 C, 5,000 x g for 30 min. and the supernatant used for DNA, RNA, protein and carbohydrate (CHO) analysis. The DNA analysis was performed using the Dische⁵ diphenylamine reagent method with purified sperm DNA as a standard. The orcinol method of Schneider (25) was used for RNA quantitation based on a standard of purified liver s-RNA. Protein was quantitated by the Cowgill and Pardee (3) method utilizing bovine serum albumin (Fraction V) as a standard. The anthrone reagent method of Morris (16) was used for carbohydrate determinations with glucose as the standard. Poly-B-hydroxybutyrate (PHB) was determined by the method of Law and Slepecky (11) using purified PHB as the standard.

Results

Growth. The responses of the bacteria to varied oxygen concentrations and the pesticide aldrin are shown on Figure 1 and were used as controls for comparative purposes throughout the remainder of the investigation. Cells grown under 8.9 mg D.O./l exhibited a short lag period (7-8 hr) and reached the stationary phase of growth after 19 hr. The culture grown under 8.9 mg D.O./l and 0.65 mg aldrin/l had an extended lag period (15-16 hr) but attained a higher final absorbancy. The cultures grown under 1.6 and 0.8 mg D.O./l exhibited a lag phase similar to the 8.9 mg D.O./l control but showed a marked increase in final absorbancy of the culture.

Samples were removed from the various phases of growth for estimation of cell numbers. Optical densities and generation times of the cultures were determined from these data and from the recorder chart and are given in Table 1. The control culture had the lowest concentration of cells as measured by viable cell counts and optical density and exhibited the longest generation time (3.0 hr). Cells grown in the presence of 0.65 mg aldrin/l had a shorter generation time and a higher cell yield than the control. Optimal growth conditions were reached when cells were cultured under 1.6 mg D.O./l.

Samples were removed through the growth phases and subjected to dry weight, total cell numbers (Petroff-Hauser), and cell size analyses (Table 2). The control cells (Samples 1-5, Table 2) showed a 24 fold decrease in dry weight and a 25 percent decrease in cell size as the organisms progressed from the lag phase to the stationary phase of growth. Cells grown in the presence of 0.65 mg aldrin/l and under 8.9 mg D.O./l

(Samples 6-10) showed a 50 fold decrease in cell weight during the growth cycle and the cell size decreased 40 percent between the late log and stationary phases of growth. Samples 11-15 (1.6 mg D.O./l) showed a slight decrease in cell weight between the late log and stationary phases and the cell size of these organisms decreased sharply (30 percent) between the late log and stationary phases. Cells grown under the lowest (0.8 mg/l) D. O. concentrations (Samples 16-20) decreased 100 fold in cell weight and decreased 18 percent in cell size between the late log and stationary phases.

Biochemical parameters. The percent dry weights as DNA of cultures grown under conditions shown in Figure 1 using aldrin as the pesticide are given in Figure 2. During the lag phase of growth each of the cultures contained about 10 percent of its dry weight as DNA. During the early log phase of growth, the culture grown under 1.65 mg D.O./l showed an increased nuclear content. This same effect was seen in cultures grown in the presence of aldrin but at a slightly later time period (mid log). All cultures showed a reduced nuclear content during the late log and stationary phases of growth.

The percent dry weights as RNA of cultures grown under conditions shown in Figure 1 using aldrin as the pesticide are given in Figure 3. During the early log phase of growth, a significant increase in RNA was observed in cultures grown under 0.8 mg D.O./l. The percent dry weight of the control cells as RNA gradually increased from the mid log to the stationary phase of growth. During the stationary phase of growth, cells,

grown under 1.6 mg D.O./l contained 57 percent of their dry weight as RNA whereas cultures grown in the presence of aldrin contained 16 percent of their dry weight as RNA.

The percent dry weight as soluble protein of cultures grown under conditions shown in Figure 1 using aldrin as the pesticide are shown in Figure 4. The first significant increase in soluble proteins is reflected in the organisms grown under 0.8 mg D.O./l during the early logarithmic phase of growth. The protein content of the cells increases from the mid log through the stationary phases of growth for all cultures except the 0.8 mg D.O./l grown cells in which there is a slight reduction in soluble protein (late log) and then a minor increase during the stationary phase.

The percent dry weight as carbohydrate (CHO) of cultures grown under condition shown in Figure 1 using aldrin as the pesticide are shown in Figure 5. The carbohydrate content of the cells showed minor fluctuations until the mid log phase of growth when the 0.8 mg D.O./l grown cells showed a small increase. Bacteria grown under the other three conditions showed a slight increase in carbohydrate content in the late log phase of growth. During the stationary phase of growth the carbohydrate content of all the cells showed a slight increase.

Bacteria harvested from the stationary phases of growth as shown in Figure 1 were analyzed for content of poly- β -hydroxybutyrate (PHB). Cells grown under control conditions contained the smallest concentration of PHB (Table 3). Cells grown in the presence of 0.65 mg/l of pesticide or under 1.6 mg D.O./l contained 7-8 times as much PHB as the control

cells. Cells grown under the lowest oxygen tension contained the highest concentration of PHB positive material.

Discussion

The pesticide used in this investigation was the chlorinated hydrocarbon aldrin. This compound was found in Lake Erie in varying amounts and may be associated with different particulates in the water (21). The pesticide had an effect on the growth and synthetic capacity of the Pseudomonas sp studied.

Bacteria grown in the presence of aldrin (0.65 mg/l) under high oxygen (8.9 mg/l) concentration had a decreased generation time and an increased cell yield compared to the control.

The cell size of the organisms was altered during the growth cycle. The cells which were enlarged during the lag phase (3.28 μ m) probably due to swelling prior to the initiation of cell division decreased in size during the early log growth phase. The control culture increased in size during the logarithmic phase of growth and decreased in size during the logarithmic phase of growth and decreased as the cultures entered the stationary phase. This pattern was followed in the cultures grown under different environmental conditions, e.g., the addition of aldrin or reduction of O_2 , but the decrease in size during the stationary phase of growth was the significant difference. The control culture decreased 13 percent in size between the late log and stationary phases while the aldrin treated cells decreased 39 percent during the same time period. The 1.6 mg D.O./l grown cells decreased 30 percent while the 0.8 mg D.O./l grown cells decreased 19 percent in size between the late log and stationary phases of growth. The decrease in cell size is attributed

to a change in the physiological or metabolic state of the cell (24). The data observed in the experiments suggest that the bacteria grown in the presence of aldrin or lowered dissolved oxygen concentration were metabolically more active and reflected a greater change in cell size when metabolism decreased on entrance to the stationary phase of growth.

The DNA, RNA, protein, and carbohydrate composition of the aldrin treated or reduced oxygen grown organisms revealed higher values than normally reported for bacteria grown free of such additives. Schaechter, et al., (24) reported that Salmonella typhimurium contained between 3 and 12 percent of the cell dry weight as DNA, dependent on the phase of growth. Morse and Carter (17), and Berner and Cohen, (1) suggested that about 3-4 percent of the cell dry weight of E. coli was DNA. The control culture in this study varied between 3 and 10 percent of cell dry weight. The treated (aldrin or lowered O₂) organisms varied between 3 and 20 percent and 2 and 21 percent for the aldrin grown and 1.6 mg D.O./l cultured cells respectively. The total DNA content of treated cells was increased significantly during the early log and mid log samples and appears to indicate increased nuclear activity during this time period. The mechanism for this is not clear at this time.

On the average about 10 percent of the cell dry weight is RNA (24). The RNA in the control cells reached a maximum of 42 percent during the late log phase of growth. The situation appeared to be different in the case of the aldrin treated or O₂ depleted cells. In aldrin samples RNA showed a more gradual increase up to the late log stage and a slight decrease in the stationary phase. The low level (1.6 mg D.O./l) grown bacteria exhibited the largest amount of RNA during the stationary portion of the growth cycle.

The protein content of all the cells correlated well with the ribosome (RNA) content throughout the experiment. Protein can compose 50 percent of the cell's dry weight (1), and the control bacteria in this study contained as much as 46 percent of their dry weight as protein. The cells grown under the different environmental conditions did not exceed the maximum amount of protein found in the control cells but did contain more protein per cell during various phases of the growth curve (early log versus stationary or mid log phases). In the case of soluble protein it appears that the presence of the pesticide or the reduced O_2 level did alter the synthetic patterns of the bacteria in a manner similar to the alterations of the RNA patterns.

The carbohydrate content of bacterial cells varies greatly but usually increases during the later phases of the growth curve. The amount of polysaccharides in *Aerobacter* and *Salmonella* (13) varied from less than 10 to over 30 percent of the cell dry weight depending on the phase of growth and concentration of sugars in the growth medium. The maximum concentration of carbohydrates is generally in the early stationary phase. These data are compatible with the results obtained during this investigation. The amount of carbohydrates gradually increased throughout the growth cycle and reached a maximum during the stationary phase of growth for all cultures. There did not appear to be any significant alteration in the synthetic patterns of CHO production.

Lower dissolved oxygen concentration or the presence of aldrin stimulated PHB production in the cultures. Chemical analysis of the cells harvested during the stationary phase of growth indicated that the bacteria grown in the presence of aldrin or 1.6 mg D.O./l contained 7-8 times more PHB than the control cells. The bacteria cultured under the lowest dissolved oxygen concentration (0.8 mg/l) contained 24 times the quantity of PHB per cell as compared to the control. This appears to be in accord with the findings of Macrae and Wilkinson, (1958), who showed PHB formation to be more favorable under limiting oxygen concentrations.

A strong interrelationship between the presence of the chlorinated pesticide and dissolved oxygen concentrations appears to exist. It seems that either lowering the amount of available oxygen or the addition of aldrin caused a stimulation in the growth rate of cultures of Pseudomonas sp and resulted in an increased yield of cellular material. It is probable that the pesticide is not acting as a carbon source but is effecting the basic metabolism of the bacteria in a way similar to the effect of lowering the available oxygen. These cyclodiene compounds are not readily oxidized or metabolized by microorganisms and except for the loss of the chemical through volatilization or adsorption to the glass vessel the presence of the compound as a non-soluble particulate in the medium must be reasonably stable. If aldrin enters the cell through a solubility in the fatty acid containing cell wall then the fate of action and mechanism of the pesticide must reside at some basic level probably involved with energy generation or use. The patterns of synthesis of RNA and soluble protein were the most severely affected by the addition of aldrin or a reduced O₂ content. Since large amounts of energy are

expended in these processes these data coincide with the above hypothesis.

The chlorinated hydrocarbon pesticides are electro-negative compounds (19), which could form a diffuse double layer on the outside of the bacterial cell in combination with mono-or polyvalent cations. These electro-negative compounds may help to concentrate ions and therefore indirectly affect the growth rate of certain microorganisms.

Floc forming microorganisms (12) and suspended microparticulates (21), have been shown to concentrate chlorinated hydrocarbon pesticides and settle to the bottom of a body of water, e.g., a lake. The microparticulates suspended in a lake also accumulate pesticides and stimulate growth of some microorganisms (22). These factors increase the pesticide concentration in the lake sediment and either alone or in concert with the lowered dissolved oxygen concentrations could greatly stimulate the growth of certain organisms in the lake ecosystem. Jannasch, (9) reported generation times in seawater of 53-60 hr for several bacteria. Assuming a similar generation time for fresh water aquatic isolates the concentration of certain pesticides and/or lowered dissolved oxygen concentrations could decrease the generation time by as much as 40 hr and effectively double the total mass of aerobic heterotrophic bacteria. These effects help to increase the already rapid eutrophication process in our lakes and other bodies of water.

Table 1. Viable cell numbers and final optical densities of cultures in the stationary phase and the generation times of the organisms in the log phase of growth.

Culture 1 Condition	Viable Cells per ml	Optical Density	Generation Time (hr)
Control 8.9mg D.O./l	5.6×10^8	0.98	3.0
0.65 mg aldrin/l 8.9 mg D.O./l	8.9×10^8	1.7	1.7
1.6 mg D.O./l	1.3×10^9	2.0	1.0
0.8 mg D.O./l	8.0×10^8	1.7	2.2

TABLE 2 Average cell size, cell dry weight, total cell numbers of *Pseudomonas* sp. at different stages of the growth cycle and grown under different conditions.

Phase of growth		cell dry wt (g x 10 ⁻¹¹ / cell)	Total cells	Cell size (μm)
Lag	(1) ^a	48	5.5 x 10 ⁶	3.28
Early Log	(2)	5	1.5 x 10 ⁸	2.60
Mid Log	(3)	4.70	1.6 x 10 ⁸	2.61
Late Log	(4)	2.20	1.0 x 10 ⁸	2.51
Stationary	(5)	1.90	9.0 x 10 ⁸	1.41
Lag	(6) ^b	44	9.5 x 10 ⁶	2.5
Early Log	(7)	21	2.9 x 10 ⁷	3.0
Mid Log	(8)	6.70	1.5 x 10 ⁸	3.65
Late Log	(9)	2.00	7.0 x 10 ⁸	3.15
Stationary	(10)	0.90	1.1 x 10 ⁹	1.51
Lag	(11) ^c	41	3.2 x 10 ⁶	3.4
Early Log	(12)	8.20	8.5 x 10 ⁷	2.71
Mid Log	(13)	2.50	2.8 x 10 ⁸	2.7
Late Log	(14)	0.79	1.4 x 10 ⁹	1.7
Stationary	(15)	0.68	1.7 x 10 ⁹	1.7
Lag	(16) ^d	48	4.5 x 10 ⁶	3.1
Early Log	(17)	2.4	4.2 x 10 ⁷	2.83
Mid Log	(18)	0.45	2.2 x 10 ⁸	3.4
Late Log	(19)	0.89	5.0 x 10 ⁸	3.11
Stationary	(20)	0.90	1.5 x 10 ⁹	2.53

^aSamples 1-5 were grown in AGS medium under
D. C. 1.

^bSamples 6-10 were grown in AGS medium under
D. C. 1 and with 0.65 mg Alarín/l.

^cSamples 11-15 were grown in AGS medium under
D. C. 1.

^dSamples 16-20 were grown in AGS medium under
D. C. 1.

Table 3. Concentration of PHB in stationary phase cells grown under different environmental conditions

Culture Condition	PHB (g/ml)	g PHB/ mg cells
Control 8.9 mg D.O./l	0.55	0.32
0.65 mg aldrin/l 8.9 D.O./l	2.20	2.20
1.6 mg D.O./l	2.65	2.30
0.8 mg D.O./l	6.70	7.44

Literature Cited

1. Berner, H. and S. S. Cohen. 1956. Synchronization of division of a thymineless mutant of Escherichia coli. J. Bacteriol. 72: 115-123.
2. Chacko, E. I., and J. L. Lockwood. 1967. Accumulation of DDT and dieldrin by microorganisms. Can. J. Microbiol. 13: 1123-1126.
3. Cowgill, R. W. and A. B. Pardee. 1957. Folin-Ciocalteu for Protein, p. 176. In Experiments in Biochemical Research Techniques. John Wiley & Sons, Inc., London.
4. Dalton, H. and J. R. Postgate. 1969. Effect of oxygen on growth of Azotobacter chroococcum in batch and continuous culture, J. Gen. Microbiol. 54: 463-473.
5. Dische, Z. 1955. Color reactions of nucleic acid components, p. 285-305. In E. Chargoff and J. N. Davidson (ed.), The Nucleic Acids, Vol. 2. Academic Press, Inc., New York.
6. Eno, C. F. and P. H. Everett. 1958. Effect of soil applications of 10 chlorinated hydrocarbon insecticides on soil microorganisms and the growth of the Stringless Black Valentine beans. Soil Sci. Soc. Am. Proc. 22: 235-238.
7. Fletcher, D. W. and W. B. Bollen. 1954. The effects of aldrin on soil microorganisms and some of their activities related to soil fertility. Appl. Microbiol. 2: 349-354.
8. Gottlieb, S. F. 1965. Hyperbaric oxygenation. Advan. Clin. Chem. 8: 69-139.
9. Jannaseh, H. W. 1969. Estimation of bacterial growth rates in natural waters J. Bacteriol. 99: 156-160.
10. Ko, W. H. and J. L. Lockwood. 1968a. Conversion of DDT to DDD in soil and the effects of the compounds on soil microorganisms. Can. J. Microbiol. 14: 1069-1073.
11. Law, J. R. and R. A. Slepecky. 1961. Assay of poly-B-hydroxybutyric acid. J. Bacteriol. 82: 33-36.
12. Leschniowsky, W. 1970. Adsorption of the chlorinated hydrocarbon pesticides aldrin by microbial floc and lake sediment and its ecological implications. Thesis. The Ohio State University.
13. Levine, S., J. H. R. Stevenson, E. C. Tabor, R. H. Bordner, and L. A. Chambers. 1953. Glycogen of enteric bacteria. J. Bacteriol. 66: 664-670.

Literature Cited (page 2)

14. Macrae, R. M. and J. F. Wilkinson. 1958. Poly- β -hydroxybutyrate metabolism in washed suspensions of Bacillus cereus and Bacillus negaterium. J. Gen. Microbiol. 19: 210-222.
15. Moore, B. and R. S. Williams. 1911. The growth of various species of bacteria and other microorganisms in atmospheres enriched with oxygen. Biochem. J. 5: 181-187.
16. Morris, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science 107: 234-235.
17. Morse, M. L. and C. E. Carter. 1949. The synthesis of nucleic acids in cultures of Escherichia coli, strains B. and B/R. J. Bacteriol. 58: 317-326.
18. Nashif, S. A. and F. E. Nelson. 1953. The lipase of Pseudomonas fragi. II. Factors affecting lipase production. J. Dairy Sci. 36: 471-480.
19. Okey, R. W. and R. H. Bogan. 1965. Apparent involvement of electronic mechanisms in limiting the microbial metabolism of pesticides. J. Water Poll. Cont. Fed. 37: 692-712.
20. Pfister, R. M., P. R. Dugan, and J. I. Frea. 1968. Methods for separation and examination of microscopic particulate fractions in water and the relationship to aquatic microflora. Abstracts, Eleventh Conf. on Great Lakes Research, p. 58-59.
21. Pfister, R. M., J. I. Frea, P. R. Dugan, K. Zaebst, J. Duchene, R. Kennedy and T. McNair. 1970. Lake Erie Microparticulate, pesticide, and microorganism interactions. Proc. XIII. Conf. Great Lakes Res., Buffalo.
22. R. M. Pfister, P. R. Dugan, and J. I. Frea. 1969. Microparticulates. Isolation from water and identification of associated chlorinated pesticides. Science 166: 878-879.
23. Reddy, M. C., D. D. Bills, and R. C. Lindsay, 1969. Ester production by Pseudomonas fragi II. Factors influencing ester levels in milk cultures. J. Appl. Microbiol. 17: 779-782.
24. Schaechter, M., O. Maale, and N. O. Kjeldgaard. 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of Salmonella typhimurium. J. Gen. Microbiol. 19: 592-606.
25. Schneider, W. C. 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of deoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. 161: 293-303.

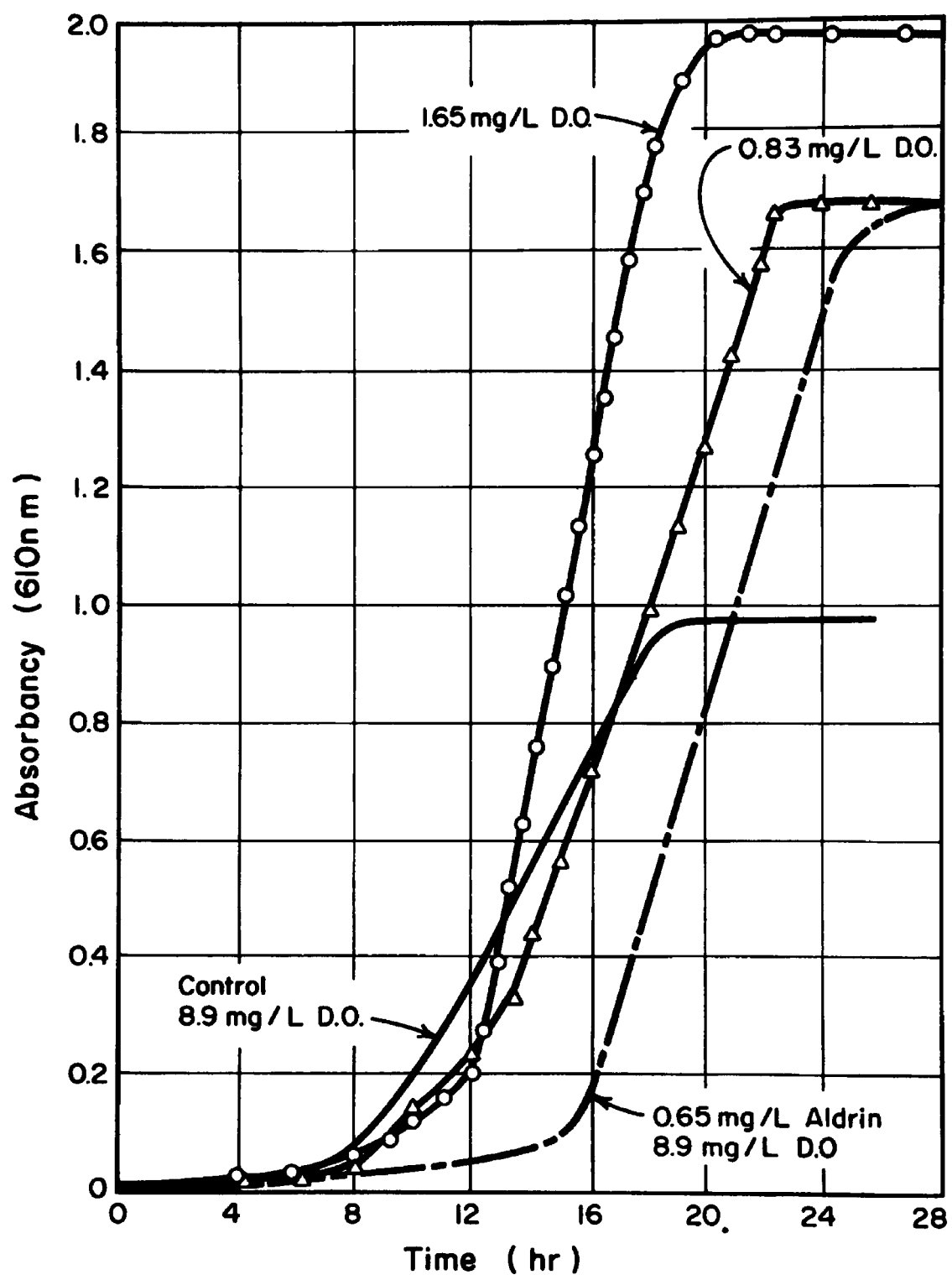


Figure 1

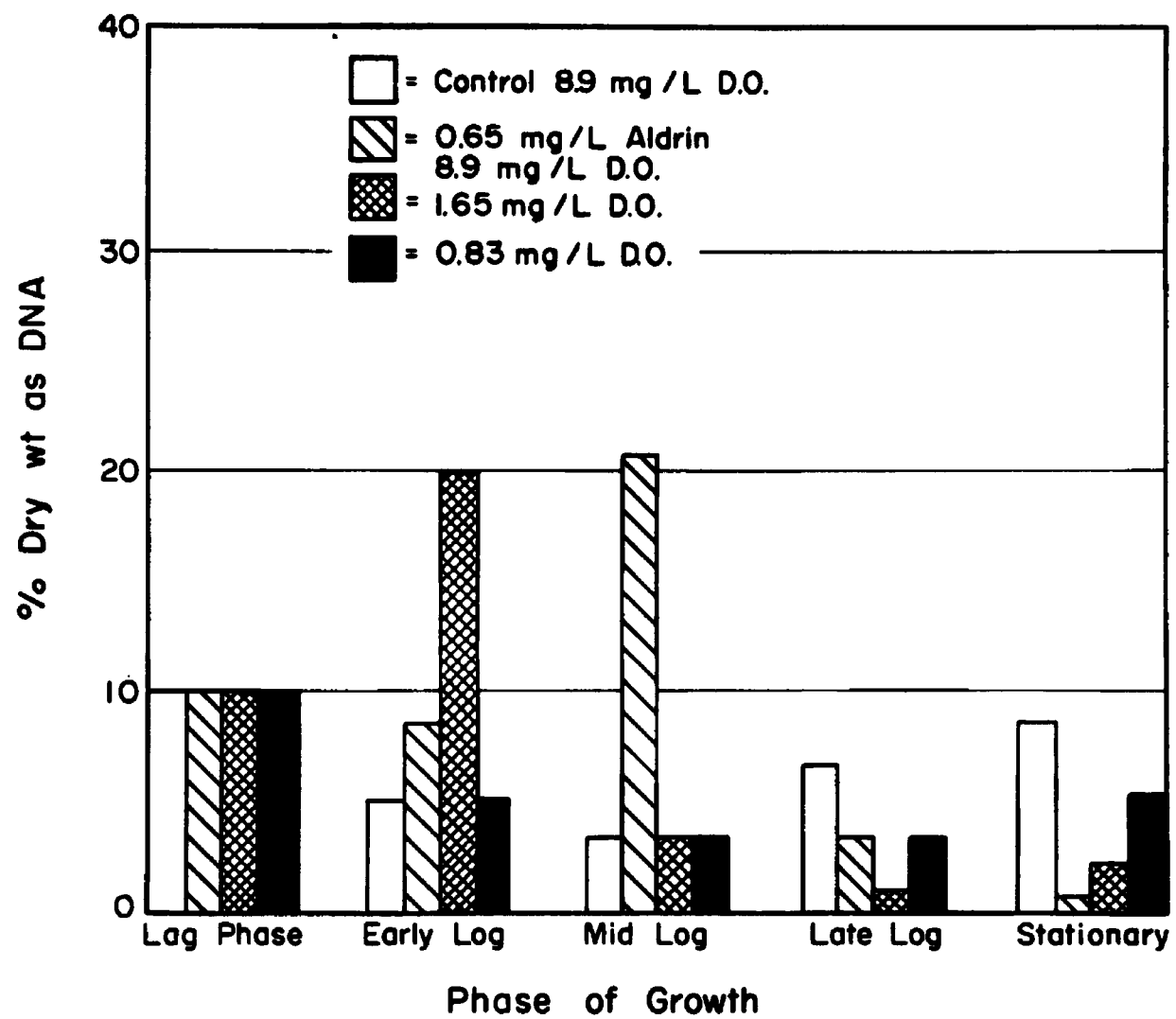


Figure 2

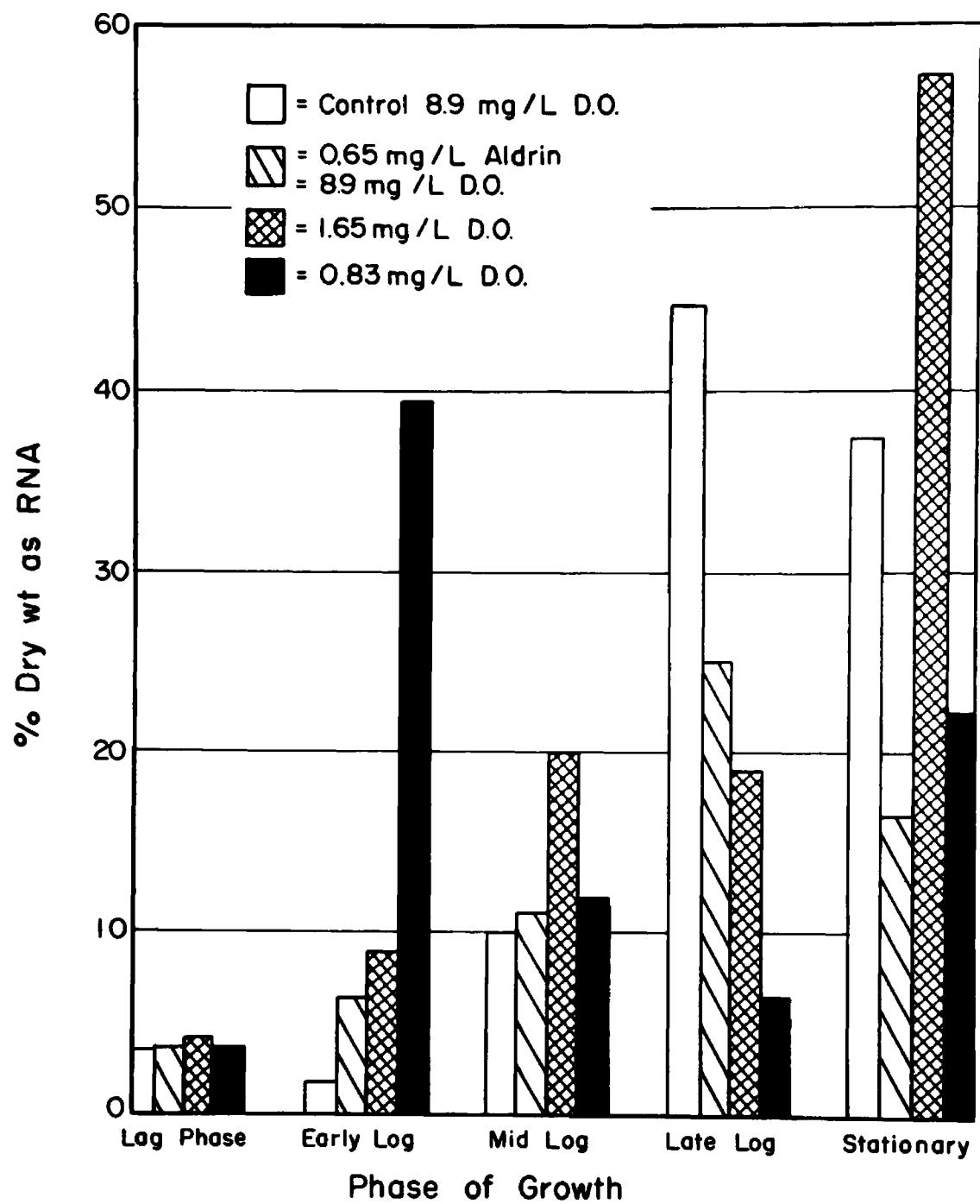


Figure 3

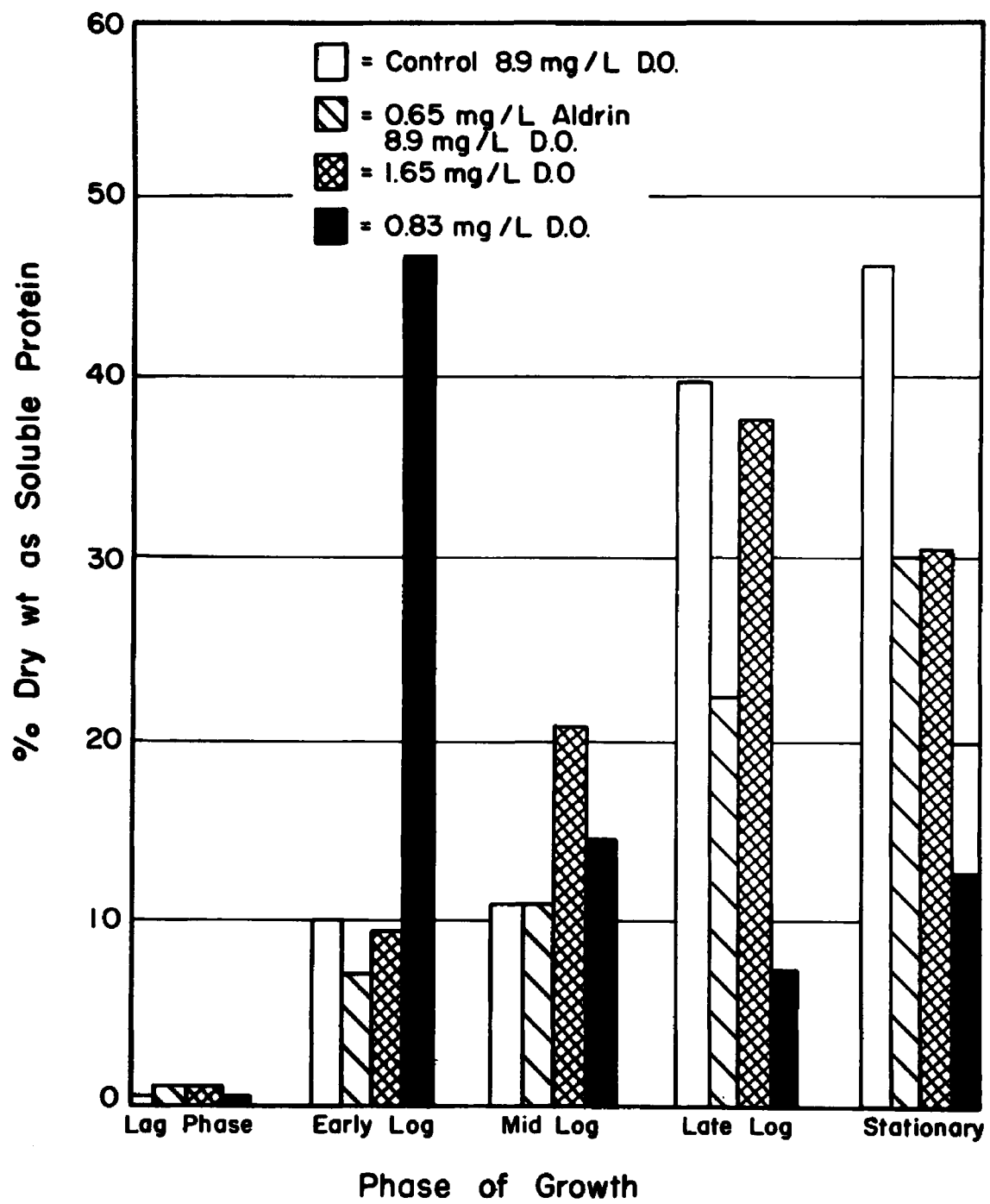


Figure 4

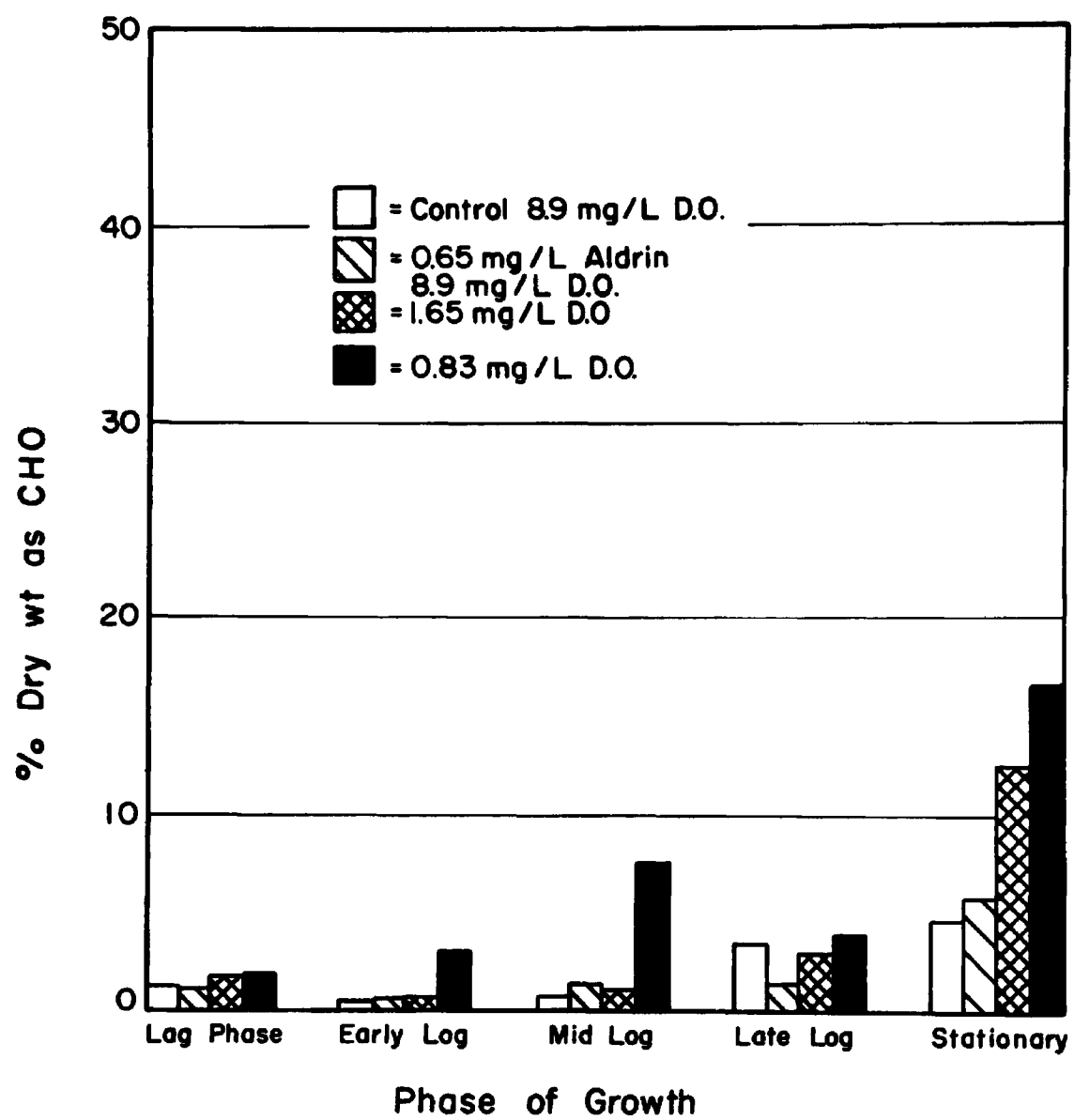


Figure 5

PART C

MICROPARTICULATES: ISOLATION FROM WATER AND IDENTIFICATION
OF ASSOCIATED CHLORINATED PESTICIDES

R. M. PFISTER, P. R. DUGAN, AND J. I. FREA
THE OHIO STATE UNIVERSITY

Abstract

Microparticulates suspended in lake water were collected by continuous centrifugation and either examined directly or placed on a linear sucrose gradient. Total residue as well as fractions of the centrifuged gradient were extracted with hexane and examined by gas chromatography for the presence of chlorinated hydrocarbon pesticides. Hexane extracts of total residues were also examined by thin-layer chromatography. Lindane and endrin were shown, by gas-liquid chromatography and thin-layer chromatography, to be associated with microparticles of different densities, when gas-liquid chromatography was used, although concentrations were below the detection limits required for confirmation by thin-layer chromatography. Samples taken at different times from different locations in Lake Erie revealed different associations with hexane-soluble electron-capturing compounds.

Introduction

The ubiquity of pesticides and the significance of their presence in the aquatic habitat has been established. Techniques for identification of pesticides in natural water usually involve extraction from water by adsorption on activated carbon. This particular method has been included in the 1962 USFHS drinking water standards as the method for the determination of organic substances in water (1). In this procedure, up to 5,000 gallons (about 19,000 liters) of water are passed through a column of activated carbon 18 by 3 inches (45.7 by 7.6 cm). The column is extracted with chloroform to remove adsorbed pesticides, and the extract is analyzed by gas chromatography. It is possible to obtain analyses by extraction of small volumes of water (for example, 1 liter) where concentrations of pesticides are high enough to cause a detector response at that level. Water samples containing pesticide concentrations below limits of detection are usually extracted by a countercurrent batch process or by liquid-liquid extraction processes to increase sensitivity of the method (2). Combinations of these methods have been useful for detection of extremely small amounts of contaminants but are quite time-consuming.

We have investigated the association of chlorinated hydrocarbon pesticides with microscopic particles suspended in Lake Erie. Water was collected in the vicinity of the Bass Islands in the western basin of Lake Erie from 15 feet (4.6 m) below the surface. A 5-gallon sample was centrifuged in a Sorvall RC-2B Szent-Györgyi and Blum continuous flow

centrifuge at 27,000g at a flow rate of 11 ml/min. The particulates from this fraction (0.15 μ m in diameter and above) were placed on top of a preformed linear gradient of sucrose (0 to 65 percent) and centrifuged at 1500g for 60 minutes. The tube was divided into four fractions, each of which was extracted with hexane. Raw water samples (2 liters) have been extracted by using the liquid-liquid extraction procedure. No detectable chlorinated pesticides were found. Analysis of the hexane extracts for presence of chlorinated hydrocarbon pesticides was made by using an Aerograph 200 gas chromatograph equipped with an electron-capture detector [250 mc of titanium tritride, column temperature 190°C, detector 200°C, injector port 230°C, 5-foot glass, 1/8 inch (internal diameter) column packed with Chromosorb W 60/80 mesh, coated with 5 percent Dow silicone SE-30, high purity N₂ carrier gas 60 ml/min].

Because gas-liquid chromatography (GLC) retention time and reinforcement of peaks are analytically inadequate for identification purposes, the presence of specific pesticides must be regarded as presumptive. The data suggested, however, that concentrations of specific pesticides associated with individual particulate fractions were below the level of detection by thin-layer chromatography (TLC) (3). Therefore, the use of confirmatory TLC on particulates from larger water samples was employed. For this purpose, a 20-gallon water sample was centrifuged as previously described. The total particulate residue was extracted with hexane and concentrated to 0.5 ml by evaporation. Aliquots (2 μ l) were injected into the gas chromatograph and the remaining volume was added as a single spot onto Eastman Chromogram 6061 TLC sheets. The TLC sheets were pre-conditioned and developed by the methods of Kovacs (4), and sprayed with 0.05 percent Rhodamine B (3); 5 μ g and 50- μ g spots of control pesticides

were chromatographed on the same TLC sheet.

The TLC sheets indicated the presence of spots with R_F values identical to lindane and endrin. The GLC recordings verified the presence of lindane and endrin as well as several additional peaks from this particular sample.

Pesticides which have been tentatively identified from GLC recordings taken from particulate fractions are lindane, heptachlor, aldrin, and endrin; there were also several unidentified peaks. Pesticides were tentatively identified by comparison of retention times to those of control samples of known purity (dielddrin, aldrin, endrin, 99+ percent, Shell Chemical Co.; DDT and isomers, 99+ percent, USPHS pesticide repository; lindane, heptachlor, 99+ percent, City Chemical Corp.), and also by reinforcement of peaks by addition of known compounds. The distribution pattern of pesticides with different particulate fractions illustrates that various chlorinated hydrocarbons have an affinity for particulates separable by density-gradient centrifugation (that is, different particles). The distribution of these pesticides demonstrates their association with the particulate compounds suspended in the water. The utility of the collection and fractionation procedures is verified by the variety of pesticide separations obtained through its use.

Table 1 is a compilation of pesticide distribution and concentration in the particulate fractions of three separate lake water samples taken at different times, based upon GLC data without TLC confirmation, since the concentration of pesticide in gradient fractions is below the limit of TLC detection.

Table 1. Nanograms of chlorinated hydrocarbon pesticides per liter of lake water associated with each fraction of particulates. Samples 79 and 75 show the qualitative presence or absence of chlorinated pesticides in each extracted fraction of two other samples taken at different times of the year.

Pesticide	Gradient fraction			
	1	2	3	4
<i>Sample 86</i>				
Lindane	0.53	4.6	3.2	16.5
Heptachlor	0.69	0	1.7	2.1
Aldrin	14.7	2.0	1.0	1.3
Endrin	9.6	0	0	0
DDT: metabolic products or isomers	0	0	0	0
<i>Sample 79</i>				
<i>p,p'</i> -DDD	+	+		+
Lindane	+		+	
Heptachlor	+	+	+	+
<i>p,p'</i> -DDT	+		+	
<i>o,p'</i> -DDT		+		
Endrin		+		+
<i>o,p'</i> -DDD			+	
<i>Sample 75</i>				
<i>o,p'</i> -DDD	+	+	+	
<i>p,p'</i> -DDD	+	+	+	+
<i>o,p'</i> -DDT	+	+	+	+
<i>p,p'</i> -DDT		+	+	+
Lindane		+		+
Heptachlor		+		+
Endrin			+	

Evidence from this study suggests that there is a quantitative as well as qualitative distribution of pesticides associating with varied particulate components in natural waters. Pesticides that were determined were attached to particles of 0.15 μm or larger in size, and different pesticides were shown to be associated individually with particles of different density. For example, in sample 86, lindane was found in greater concentration in fraction 4, the inorganic portion of the particulate material (5), while aldrin and endrin were associated with less dense upper fractions 1, 2, and 3, which consist primarily of organics, detritus, and microorganisms. The presence of various chlorinated pesticides was associated with different particulate compounds of the environment. These compounds could be present in the environment as either molecular aggregates in aqueous solution (4.1 nm or less) or in suspension (up to 0.11 μm) (6). In our system, particulates below 0.15 μm were not included, so that the presence of pesticides is indicative of some kind of true pesticide-particulate association. The particles could be cell, detritus, or inorganic materials (for example, clay minerals).

Different chlorinated hydrocarbons are present in different water samples. For example, samples 70 and 75 suggest the presence of DDT, its isomers, and metabolic breakdown products, whereas no DDT-group compounds were detected in sample 86. The lack of detection of DDT or isomers in this sample may be a reflection of the possibility of the specific association of such compounds with specific particulates, or, of course, it may be that these compounds were below the level of detection in the quantity of particulate used for the extraction.

In analyses where activated carbon filters have been used (for example, U.S. Public Health Service Water Pollution Surveillance Program) levels (0.05 ng to 0.05 ug/liter) of pesticides are detected. This technique has made use of 30-mesh and 4-by 10-mesh carbon which has been preextracted to remove organic material. It seems likely that chlorinated hydrocarbons associated with particles that have the general size of 0.15 um could pass through coarse carbon filters and remain undetected in the water sample. Evidence from our study suggests that removal and analysis of particulates may need to be included to give more adequate estimates of pesticides in aquatic environments. In an environment such as Lake Erie, where the shallow water is so easily disturbed by wind action, the turnover and accumulation of pesticides in bottom sediments may be significant.

References

1. H. P. Burchfield and D. E. Johnson, Guide to the Analysis of Pesticide Residues, U. S. Department of Health, Education, and Welfare, Public Health Service Bureau of State Services (Environmental Health) (Government Printing Office, Washington, D. C., 1965).
2. L. Kahn and C. H. Wayman, Anal. Chem. 36: 1340 (1964).
3. A. W. Breidenback, J. J. Lichtenberg, C. F. Henke, B. J. Smith, J. W. Eighelberger, Jr., H. Stierli, The Identification and Measurement of Chlorinated Hydrocarbon Pesticides in Surface Waters, U. S. Department of Interior, Federal Water Pollution Control Administration, 17-22 (Government Printing Office, Washington, D. C., 1966).
4. M. F. Kovacs, J. Assoc. Off. Agr. Chem., 46: 884 (1963); *ibid.* 49: 365 (1966).
5. W. T. Lammers, Limnol. Oceanogr., 7, 224 (1962).
6. M. C. Bowman, F. Acree, Jr., M. K. Corbett, J. Agr. Food Chem., 8, 406 (1960).
7. Supported in part by grants A-006-OHIO and O13 Ohio, Office of Water Resources Research, U. S. Department of Interior, Mrs. Patricia Kennedy and Mrs. Virginia Price contributed technical assistance.

PART D

ALDRIN: REMOVAL FROM LAKE WATER BY FLOCCULENT BACTERIA

W. O. LESHNIOWSKY, P. R. DUGAN, R. M. PFISTER,
J. I. FREA, AND C. I. RANGLES
THE OHIO STATE UNIVERSITY

Abstract

Floc-forming bacteria isolated from Lake Erie adsorb and concentrate aldrin from colloidal dispersion so that the settling of the bacterial flocs removes aldrin from the water phase. Contemporary sediments forming in Lake Erie contain aldrin and could adsorb more. The sediments consist of a conglomerate floc of bacteria, diatoms, and inorganic and detrital particles. Flocculent bacteria also adsorb microparticulates, and this adsorption capacity represents a mechanism for sediment formation and for the removal of suspended particles including aldrin from the water column.

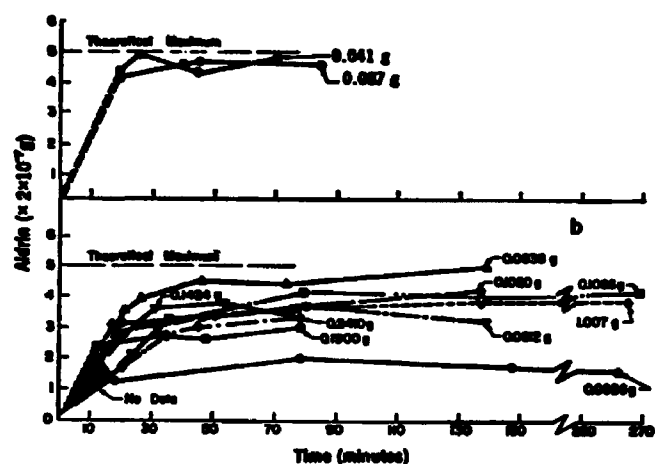
Introduction

Many chlorinated hydrocarbon insecticides have been isolated from surface waters, usually in concentrations of less than 1 ug/liter or 1 part per billion (ppb). The deleterious effects of pesticide in water have been established (1). Our interest is in the fate of these chemicals in a water column and particularly in their adsorption to silt- and floc-forming bacteria which form contemporary sediment in lakes. Bacterial floc is an aggregation of cells which results in a macroscopic bacterial clump that settles from the liquid, thus leaving that medium less turbid. This type of growth appears to result from physical, chemical, and biological interactions when extracellular fibrillar polymers are synthesized by organisms (see 2).

Our study of aerobic bacteria isolated from Lake Erie revealed that of 33 isolates tested in six different growth media 19 formed flocs in at least one medium, whereas ten formed flocs in two or more of the media. We report here a study of the ability of two of the floc-forming isolates to concentrate and accumulate the pesticide aldrin (3) from solution. One bacterium was an orange-red pigmented Gram-negative rod, tentatively identified as either a Flavobacterium or Protaminobacter. The other was a Gram-positive species of Bacillus.

Our experimental procedure was as follows: The test organisms were grown in a shake flask at ambient temperature ($22^{\circ} \pm 2^{\circ} \text{C}$) in nutrient broth (8 g/liter, Difco), harvested by centrifugation, washed twice with distilled water, and resuspended in 25 ml of distilled water. Erlenmeyer flasks containing 50-ml suspensions of bacterial floc were then placed on a rotary shaker and 1 ml of aldrin dissolved in acetone was added to give a final aldrin concentration of 1×10^{-6} g/ml or 1 part per million (ppm). After being shaken at 120 rev/min for the desired time period, the flasks were removed from the shaker and the floc was separated from the supernatant by centrifugation. The flocs were washed twice with distilled water and the washings were added to the original supernatant. The pesticide exposure time was calculated as that period between the addition of aldrin to the solution and the separation of the second washing from the bacterial floc. The floc and supernatant fractions were extracted separately with a mixture of heptane and acetone (3:1, by volume). The organic solvent fractions containing the aldrin were concentrated by evaporation and adjusted to a volume of 4 ml. Samples (2 μl each) were injected into a gas chromatograph (Aerograph model 200) fitted with an electron capture detector (4).

The total amount of aldrin adsorbed to bacterial floc as a function of time is plotted in Figure 1. The theoretical maximum for aldrin adsorption calculated from a standard curve is 1 ppm. The recovery values for aldrin varied in individual experiments between 70 and 130 percent (0.7 to 1.3 ppm), with the variation possibly due either to adsorption on glassware (5) or to the varying sensitivity of the electron capture detector. Almost all of the aldrin adsorption to floc took place



showing the initial amount of aldrin found in two different samples of contemporary sediment (silt) (broken lines) and the additional aldrin adsorbed during the experiment by the two samples as a function of time (solid lines).

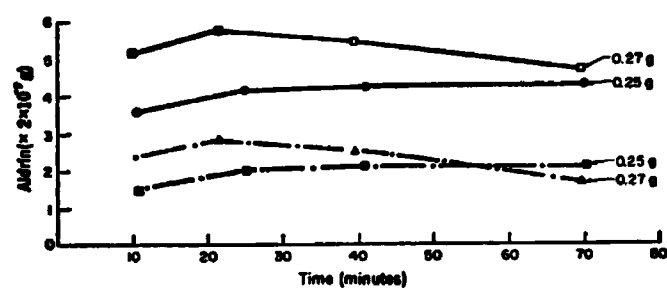


Fig. 1 (left). Curves showing adsorption of aldrin by both (a) Gram-positive bacterial floc and (b) Gram-negative bacterial floc as a function of time (solid lines). Numerals on each curve indicate the dry weight of bacterial floc used in each experiment. Broken and dashed lines indicate extrapolation from the first experimental point to zero time. Fig. 2 (right). Curves

within the first 20 minutes of contact. That the amount of aldrin adsorbed in most cases remained nearly constant after 20 minutes was verified statistically by single variable linear regression at both the 1 and the 5 percent levels of significance when the amounts of aldrin adsorbed as a function of time were compared. All of the aldrin added to the Gram-positive bacteria was recovered from the floc; none was recovered from the supernatant. The recovery value averaged 88 percent. The slope of the line is linear and $t = 0$ (6) at both the 1 and 5 percent significance levels when floc weight as a function of pesticide adsorbed was evaluated. This linearity is due to the high rate of aldrin adsorption by floc which resulted in maximum adsorption within the minimum time period required to obtain the first adsorption value (that is, 12 to 15 minutes). The fact that there was no significant difference in the amount of aldrin adsorbed by 0.041 g of floc as compared to 0.087 g of floc indicates that 0.41 g was sufficient to adsorb all of the available aldrin.

Adsorption curves shown in Figure 1 indicate a rapid uptake of aldrin during the first 20 minutes until maximum theoretical adsorption is reached. As aldrin is adsorbed, less is available in solution to be adsorbed. Therefore, the flocs effectively adsorb from more dilute solutions than anticipated by the initial test concentration. This would essentially represent adsorption from lower, more realistic, pesticide concentrations found in Lake Erie.

Data for the Gram-negative organism show that the amount of aldrin adsorbed by an equal weight of cell flocs remained the same or increased only slightly with time beyond 20 minutes and either decreased or remained unchanged in the supernatant. The adsorption curve was linear at the 1 and 5 percent significance levels, but only if the 0.0656 g value was omitted from the calculations. If this value was included, the data did not represent a linear relation at either level. However, in neither case was $r = 0$, an indication of a relationship between adsorption and floc weight in this case. Deviation from $r = 0$ results from a slower adsorption by the Gram-negative bacteria as compared to the Gram-positive bacteria.

The concentrating effect of these bacteria is considerable. For example, when 0.041 g of Gram-positive floc adsorbed pesticide from 25 g of water, the concentration factor was about 625 to 1 within 20 minutes. A similar but slightly smaller amount of adsorption occurred with the Gram-negative organism. Analogous findings have been reported for algae (7).

Samples of natural sediment that were in the process of settling and accumulating in Lake Erie were collected by specially designed sediment collectors placed on reefs (8). This sediment consisted primarily of inorganic matter. We analyzed the sediment in a manner identical to that described for bacterial floc, and we also examined the sediment on a microcoulometer (Dohrmann Instrument Company model 200A) (9).

The presence of both aldrin and dieldrin (3) in contemporary sediment was detected by both gas chromatography and microcoulometry. Additional aldrin added experimentally was absorbed and, as shown in Figure 2, the concentrations after 10 and 70 minutes were almost equal. No aldrin was detected in the supernatant. The data were linear at both the 1 and 5 percent levels of significance and r was equal to zero.

Contemporary lake sediments appear to accumulate pesticide from suspension in a manner similar to that shown for bacterial floc. Floc-forming bacteria are common in the lake environment and experimentally have a rapid and high adsorption capacity for aldrin. Organic "floc-like" bottom sediments from the eastern basin of Lake Erie have been reported (10), and electron microscopic examination of contemporary sediments from Lake Erie shows that these sediments consist of a conglomerate of bacteria, diatoms, and inorganic and detrital particulates.

Counts of 10^5 aerobic and 10^6 anaerobic heterotrophic bacteria have been obtained per gram (wet weight) of contemporary lake sediment. In this regard, clay particles are known to adsorb pesticide and lake sediments are known to adsorb lindane (3,11). Pfister, et al, (12) reported that chlorinated hydrocarbons both behave as suspended microparticulates and are associated with other microparticulates including detritus in the water column. Other researchers (13) have established that DDT (3) is taken up from organic detritus by fiddler crabs. Significant concentrations of chlorinated pesticides have been detected in algae and lake bottom mud (14). It is known that actinomycetes, fungi, and other bacteria adsorb and concentrate pesticides from solution (15),

and that microparticulates associate with microorganisms (16).

Particulate organic material has been considered a potentially important source of food for filter-feeding marine organisms (17). The suggestion has been made that most of the particulate organic carbon at depths shallower than 175 m in the Atlantic Ocean off South America consists of living organisms and decomposable organic matter (18).

We conclude that floc-forming microorganisms act as adsorbants for other suspended microparticles including chlorinated hydrocarbons and that this adsorption represents a natural process for the removal of microparticles from the water column. Once the microparticles have settled from suspension, the fate of the pesticides is in question, but they may be degraded under anaerobic conditions (19). It is likely that pesticides concentrated in bottom sediments for even short periods of time would exert an insecticidal effect on the bottom insects and other susceptible fauna. Jensen and Gauvin (20) and Carlson (21) have shown that different species of stone fly and mayfly naiads have varying susceptibilities to the same pesticide. This may explain the disappearance of certain insects from Lake Erie such as mayflies, and the persistence or increase of others. The same may hold true for other organisms in the lake.

References

1. O. B. Cope, in Research in Pesticides (Academic Press, New York, 1964).
2. P. L. Busch and W. Stumm, Environ. Sci. Tech., 2: 1 (1968); B. A. Friedman, P. R. Dugan, R. M. Pfister, C. C. Remsen, J. Bacteriol. 98: 1328 (1969).
3. Aldrin: 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-endo-exo-1,4:5,8-dimethanonaphthalene; dieldrin: 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-endo-exo-1,4: 5,8-dimethanonaphthalene; lindane: 1,2,3,4,5,6-hexachlorocyclohexane; DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane.
4. Operating conditions were as follows: 250 mc of titanium tritritide, Ti^3H_3 ; column temperature, 185°C; detector temperature, 200°C; injector port temperature, 225°C; glass column 152 cm long and 0.32 cm in internal diameter packed with Chromosorb W 60/80 mesh, coated with 5 percent Dow silicone SE-30; high-purity nitrogen carrier gas; flow rate, 60 ml/min.
5. M. C. Bowman, F. Aeree, Jr., C. H. Schmidt, M. Beroza, J. Econ. Entomol. 52: 6 (1969).
6. A value of zero indicates that there is no relationship between the x- and y-axes and therefore that there is no real difference between the quantity of aldrin absorbed by 0.041 g and by 0.087 g of floc in these experiments.
7. J. E. Keil and L. E. Priester, Bull. Environ. Contamination Toxicol. 4: 3 (1969); B. D. Vance and W. Drummond, J. Amer. Water Works Assoc., 61: 7 (1969).
8. C. E. Herdendorf, Proc. Conf. Great Lakes Res., 11th, Ann Arbor, Michigan, 1968 (1968), p. 188.
9. Inlet temperature, 300°C; furnace temperature, 800°C; equipped with a glass column 182 cm long and 5 mm in internal diameter, packed with Chromosorb W 60/80 mesh, coated with 7.55 percent QF-1 and 5.20 percent DC 200; high-purity nitrogen gas; flow rate, 190 ml/min.
10. J. F. Storr and C. J. Cazeau, Proc. Conf. Great Lakes Res., 12th Ann Arbor, Michigan, 1969 (1969), p. 368.
11. E. G. Lotse, D. A. Graetz, G. Chesters, G. B. Lee, L. W. Newland, Environmental Science Tech., 2: 353 (1968).

12. R. M. Pfister, P. R. Dugan, J. I. Frea, Science, 166: 878 (1969).
13. W. E. Odum, G. M. Woodwell, C. F. Wurster, *ibid.* 164: 576 (1969).
14. W. D. Johnson, F. D. Fuller, L. E. Scarce, Proc. Conf. Great Lakes Res., 10th, Ann Arbor, Michigan, 1967 (1967), p. 363.
15. C. I. Chacko and J. L. Lockwood, Can. J. Microbiol. 13, 1123 (1967); S. Voerman and P. M. L. Tammes, Bull. Environ. Contamination Toxicol., 4: 5 (1969).
16. R. M. Pfister, P. R. Dugan, J. I. Frea, Proc. Conf. Great Lakes Res., 11th, Ann Arbor, Michigan, 1968 (1968), p. 111.
17. E. R. Baylor and W. H. Sutcliffe, Limnol. Oceanogr. 8: 369 (1963).
18. L. A. Hobson and D. W. Menzel, *ibid.* 14: 159 (1969).
19. D. W. Hill and P. L. McCarty, J. Water Pollut. Contr. Fed., 39: 1259 (1967).
20. L. J. Jensen and A. R. Gaufin, *ibid.*, 38: 1273 (1966).
21. C. A. Carlson, Trans. Amer. Fish. Soc., 95: 1 (1966).
22. Supported by grant No. B-013 from the Office of Water Resources Research, and grant No. WP-00713 from the Federal Water Quality Administration, U. S. Department of Interior. We thank E. Herdendorf of the Ohio Department of Natural Resources Geological Survey for supplying the contemporary sediment from Lake Erie.

PART E

PARTICULATE FRACTIONS IN WATER AND THE RELATIONSHIP
TO AQUATIC MICROFLORA

R. M. PFISTER, P. R. DUGAN, J. I. FREA, AND F. T. STONE
THE OHIO STATE UNIVERSITY

Abstract

This investigation was to study the interaction of environmental contaminants (defined as substances not formed biologically or naturally and which are not normally indigenous to the water) on the microbial portion of the ecosystem. Particulate suspended materials (minerals and detritus) were examined on a physical and biological basis, and characterized using differential and gradient centrifugation in conjunction with electron microscopy. Several characteristic fractions of suspended particulate material were examined for ability to influence biological reactions. The particulate fraction of water is important to microbial relationships in the area of interfaces and biological activity. It is known that particles and molecules in solution accumulate at interfaces (this includes chemicals which can either act favorably (nutrients) to organisms or unfavorably (pesticides) to organisms), and that enzymatic reactions are concentrated at membranous surfaces. Therefore, it is of significant importance to study the capabilities of non-biologicals that commonly end up in the waters on such colloidal or molecular interfacial systems.

Introduction

The natural process of eutrophication appears to be greatly accelerated in Lake Erie. The net effect of accelerated eutrophication involves stimulated shifts in predominant organisms, changes in growth patterns, and an overall biomass increase in the Lake. This could result from a variety of contributory factors; e.g. cities, agriculture, erosion, industrial wastes, heat from power plants, etc.

At the physiological level, growth of organisms can be interpreted in physical-chemical and nutritional terms. The processes then would be related to the extent and nature of growth promoting, or inhibiting, substances which enter the Lake; and therefore to streams in the watershed that carry material into the Lake. Many of the substances that enter the Lake are wastes which can be modified, via the metabolic activities of protists and other microorganisms, to serve as nutrients for higher life forms. This includes the conversion of such compounds as detergents, hydrocarbons, cellulose, chitin, etc. to either microbial cell mass which can then be utilized directly by higher forms.

Metabolic activities, hence total growth, of microbes tend to increase at interfaces (Bigger, 1941; ZoBell, 1943; Zvyagintsev, 1962). Indeed, the reason for extremely high metabolic rates in protists, as compared to that in multicellular organisms, is largely due to their high surface to volume ratio. Microorganisms being a system of surfaces composed of membranous organelles.

It is known that suspended particulates as well as dissolved chemicals tend to accumulate at interfaces (Henrici, 1935; Heukelekian, 1940; Riley, 1963; Wood, 1962). This phenomenon is the basis of foam or froth flotation recovery processes. Further, suspended particulates, when available, represent active sites for adsorption and concentration of dissolved chemicals at the surface. This is the physio-chemical basis for many types of chromatography and purification processes.

The purpose of this investigation is to examine the interaction of suspended particles and growth of microorganisms in relation to accumulation of dissolved nutrients in the Lake, with an ultimate objective of determining whether or not these processes have a significant influence on eutrophication. Particulates in the size range of 10 microns or less have been chosen for study because the high surface to volume ratio is likely to be quite significant in relation to accelerated growth rates.

Methods and Results

Figure 1 shows a flow diagram for the analysis of water. Water samples were collected from a depth of 15 ft in the Lake, or just below surface in the Sandusky River. The diagram illustrates that centrifugation was used in the process. This was done by collecting a 5-10 gallon sample. The bacterial counts and initial isolations of bacteria were done as soon as possible upon retrieval of the water sample and were frequently accomplished (weather permitting) on board the boat. Incubation was carried out at 30°C.

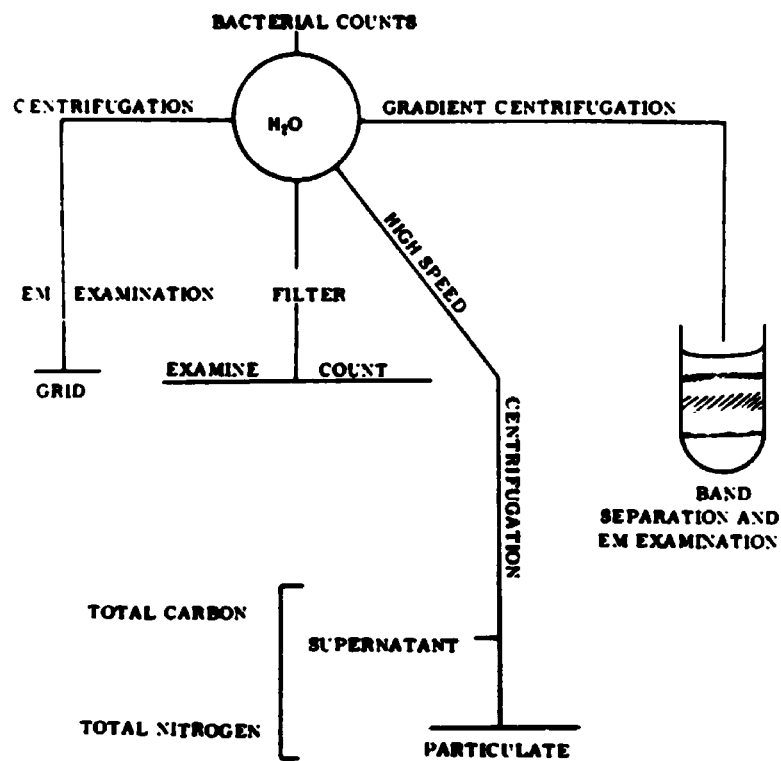


FIGURE 1. FLOW DIAGRAM OF PROCEDURE USED DURING EXPERIMENTATION.

Water was centrifuged at 27,000 x g with a flow rate of 45 ml/min in a Sorvall Szent-Gorgi continuous-flow centrifuge. This permitted the removal of particles down to 0.3 microns. The supernatant water was then passed through the centrifuge at 27,000 x g with a flow rate of 11 ml/min which removed colloids down to 0.1 micron. Solid residue from these fractionations were weighed and placed on top of a gradient constructed of sucrose with a linear density of 1.0765 to 1.2241 and centrifuged at 1,500 x g for 60 min (Lammers, 1962, 1964 and 1967).

Bands were collected by using a Beckman tube-cutting device and particulates in each band were either dialysed against distilled water or washed in distilled water by high-speed centrifugation. 0.01 ml of each fraction was placed on a carbon-coated electron microscope grid, dried and examined. Both inorganic and organic particles could be examined and estimated as to size, distribution and density (Figure 2). It can be seen that the inorganic particles, including diatoms, are found at lesser densities. Particles in this treatment will separate primarily on the basis of density.

Another method which we have employed to separate particulates on the basis of size is the direct preparation of carbon replicas on membrane filter surfaces (Millipore Corp., Bedford, Mass.).

Volumes of from 5 to 50 ml of water were pushed through the filter before it was removed and air dried. 1 mm square pieces were cut from the sample, shadowed and carbon replicated. Carbon films were washed in acetone, chromic acid and water. Figure 3 shows an electron micrograph of the surface of a standard unused 0.45 micron pore size filter.

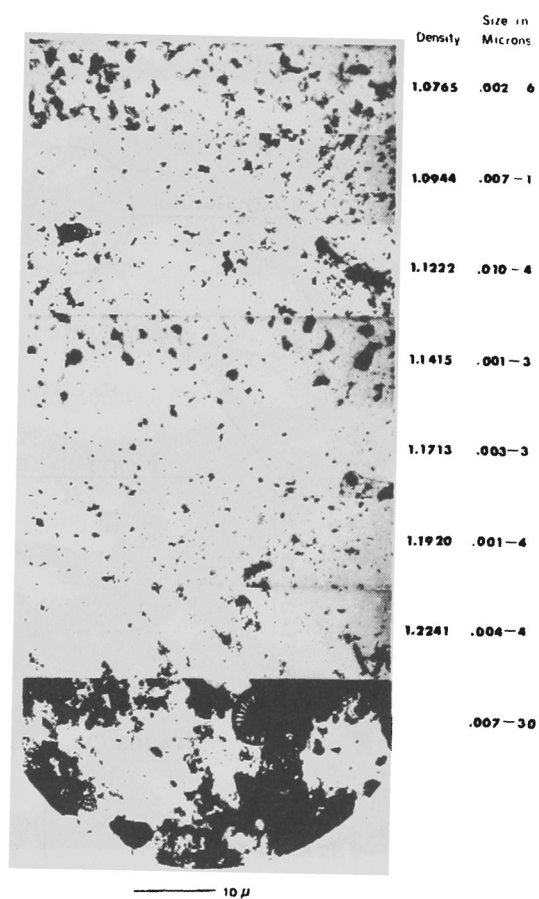


FIGURE 2. COMPOSITE PHOTOGRAPH MADE FROM THE EXAMINATION OF PARTICULATE FRACTIONS SEPARATED BY USING A LINEAR SUCROSE GRADIENT. EACH SEGMENT IN THE PHOTOGRAPH REPRESENTS A SECTION IN THE GRADIENT REMOVED WITH A TUBE-CUTTING DEVICE.

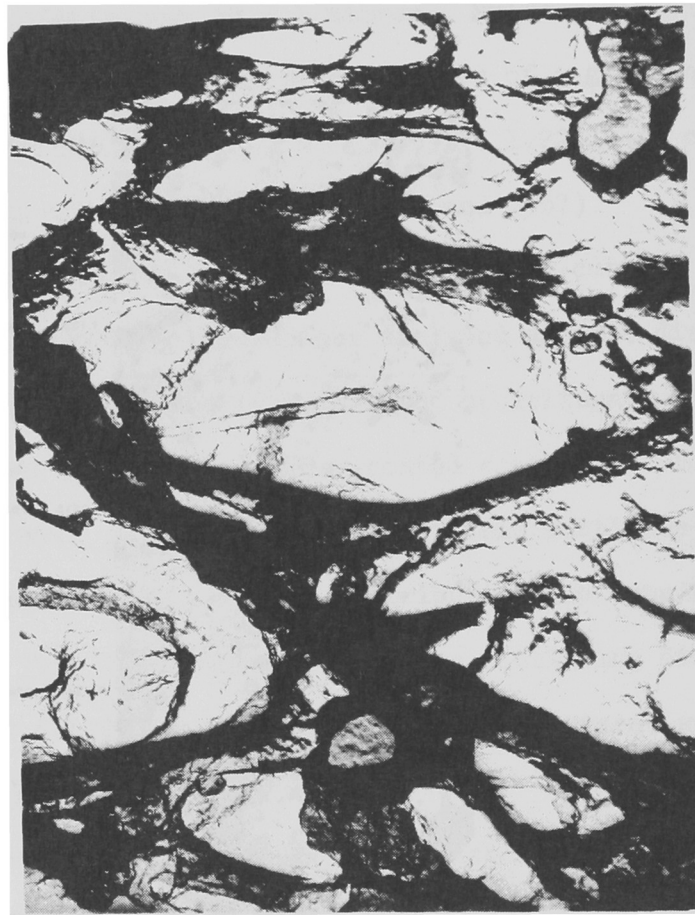
**3**

FIGURE 3. ELECTRON MICROGRAPH OF A CARBON REPLICA OF A STANDARD UNUSED .45 MICRON PORE SIZE MILLIPORE FILTER.

In Figure 4 a variety of particulates can be seen on the surface. Chromic acid in the washing process is not particularly effective against inorganics, and these can be seen as dense electron-scattering particles. The other particles, which have been removed by the treatment and are seen as the same density as the background carbon, were probably organic in origin.

To examine the biological effects of particulates which had been fractionated as described, several experiments were attempted, Table 1. Pure cultures growing on half-strength tryptone glucose yeast extract broth were subjected to the addition of particulate fractions and their supernatants. The histogram shows the growth of each culture as compared to a control designated as "no fraction" in the graph. Each bar of the histogram has been corrected for effects of turbidity caused by the addition of the particulates.

Results show that in three of the organisms examined, the particles 0.3 micron or larger were effective in inhibiting the growth as measured turbidimetrically. The growth of the yeast culture appeared to be slightly enhanced by the particulates. The explanation of these effects has not been determined.

Another experiment was conducted with pure cultures of Micromonospora sp. and Streptomyces sp. which had been isolated from Lake Erie. The particulate fraction 0.3 micron and larger was added to a salts medium which contained no carbon source. The addition of these particulates stimulated a significant increase in relative biomass over the control cultures which received no particulates. The arrow indicates

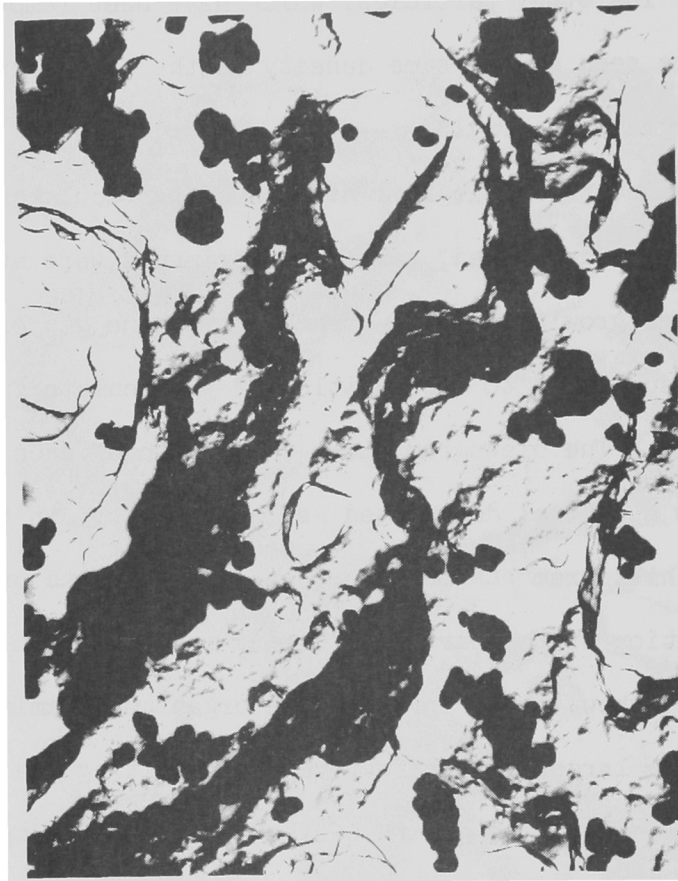
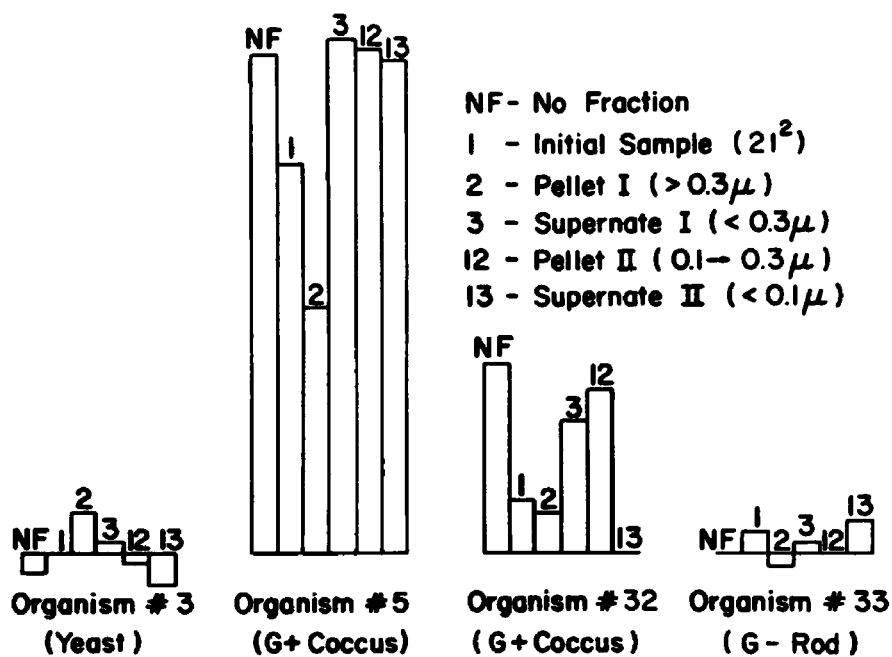
**4**

FIGURE 4. CARBON REPLICA OF A MILLIPORE FILTER AFTER
FILTERING 10 ml OF LAKE ERIE WATER. DARK
PARTICLES ARE PROBABLY INORGANIC.
30,000X

TABLE 1. RESULTS OF AN EXPERIMENT SHOWING THE EFFECT ON MICROBIAL GROWTH BY TWO PARTICULATE FRACTIONS AND THEIR SUPERNATENTS. THE BARS OF THE HISTOGRAM REPRESENT THE RELATIVE GROWTH MEASURED TURBIDIMETRICALLY AS COMPARED TO A GROWTH CONTROL (NO FRACTION). EACH BAR HAS BEEN CORRECTED FOR THE EFFECTS OF TURBIDITY CAUSED BY ADDITION OF THE PARTICULATES.



the concentration of this particulate fraction in the Lake. Results of this experiment are shown in Table 2.

Figure 5 shows the Streptomyces attached to particles in this experiment. This appears to be the development of an aggregate between suspended particles and organisms.

In a lake such as Erie, where the turbidity is very high and particulates are in the water column in high concentration, they probably play a significant role in the aging and eutrophication of the lake.

Figure 6 clearly shows the association of sub-microscopic particles of magnesium silicate (talc) with an exocellular polymer produced by this aquatic bacterium. This organism is an unidentified floc-forming pseudomonad which has been previously described (Friedman, 1968). This serve to illustrate how sub-microscopic particles can be aggregated into a larger particulate via the activity of microorganisms.

Discussion

The water in Lake Erie is relatively high in suspended particulates and this may be related to reported increases in the rate of eutrophication.

This report indicates that the particulates in the Lake are comprised of substances having a variety of densities and that fractions can be separated which exert an influence on the growth and metabolism of microbes taken from the Lake. It can therefore be concluded that specific aquatic microbes have the capacity for accumulating inorganic

TABLE 2. RESULTS OF AN EXPERIMENT SHOWING THE EFFECTS OF THE ADDITION OF A 0.3 MICRON AND LARGER PARTICULATE FRACTION ON THE GROWTH OF STREPTOMYCES SP. AND MICROMONOSPORA SP. THE GRAPH SHOWS INCREASE IN RELATIVE BIOMASS AS MEASURED BY INCREASE IN DNA CONTENT THROUGHOUT THE EXPERIMENT. THE ARROW INDICATES THE CONCENTRATION OF THIS FRACTION IN THE LAKE.

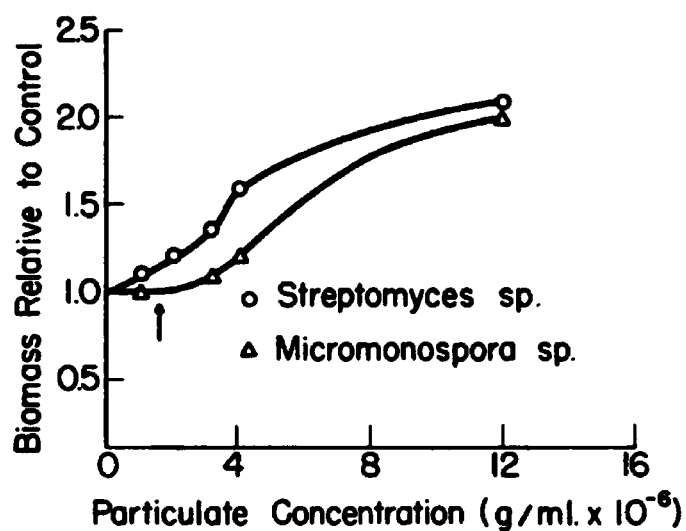




FIGURE 5. STREPTOMYCES SP. ISOLATED FROM LAKE ERIE
SHOWN ASSOCIATING WITH 0.3 MICRON AND
LARGER PARTICULATE FRACTION FROM LAKE ERIE.
THE PARTICLES IN THIS EXPERIMENT ARE THE
SOLE SOURCE OF CARBON. 1,600 X

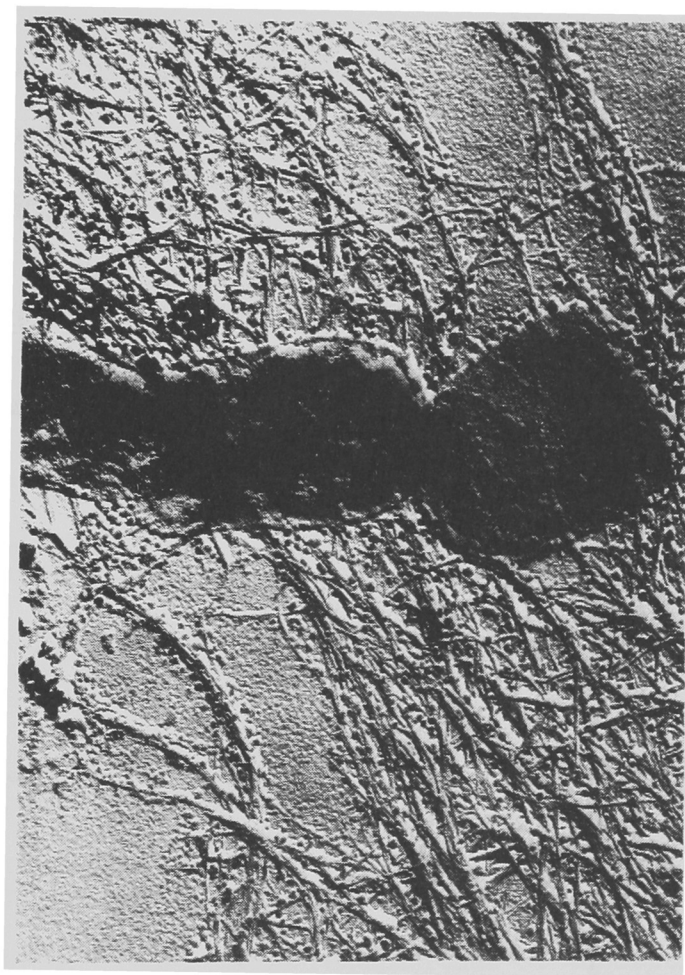


FIGURE 6. THE PHOTOGRAPH SHOWS THE ASSOCIATION OF MAGNESIUM SILICATE (TALC) WITH A POLYMER PRODUCED BY AN UNIDENTIFIED FLOC-FORMING PSEUDOMONAD. 25,000 X

micro-particulates. This in effect alters the distribution and availability of micro-particulate surfaces and may even remove them from suspension via a flocculation process. Growth of the organisms to which micro-particulates become attached is known to be controlled by available nutrients.

Postulation of a system of ecological controls can be made which involves interaction of nutrient concentration, suspended micro-particulates, and buildup of larger aggregates from association of inorganic and living particles.

References

- Bigger, J. W., and J. H. Nelson. 1941. The growth of coliform bacilli in distilled water. *J. Pathol. Bacteriol.* 53: 189-206.
- Friedman, B. A., and P. R. Dugan. 1968. Identification of Zoogloea species and the relationship to zoogloea matrix and floc formation. *J. Bacteriol.* 95: 1903-1909.
- Henrici, A. T., and D. E. Johnson. 1935. Studies of freshwater bacteria. II. Stalked bacteria, a new order of Schizomycetes. *J. Bacteriol.* 30: 61-93.
- Heukelekian, H., and A. Heller. 1940. Relation between food concentration and surface for bacterial growth. *J. Bacteriol.* 40: 547-558.
- Lammers, W. T. 1962. Density gradient separation of plankton and clay from river water. *Limnology and Oceanography* 7: 224-229.
- Lammers, W. T. 1964. Density gradient separation of plankton and clay from river water: Theory and applications. *Verhandl. Intern. Verein. Limnology* 15: 1021-1028.
- Lammers, W. T. 1967. Biophysical Limnology - Separation of suspended and colloidal particles from natural water. *Environmental Science and Technology*, Vol. I, No. 1, Jan. 1967, pg. 52-57.
- Riley, G. A. 1963. Organic aggregates in seawater and the dynamics of their formation and utilization. *Limnology and Oceanography* 8: 372-381.
- Wood, E. J. F., and C. H. Oppenheimer. 1962. Note on fluorescence microscopy in marine microbiology. *Z. f. Allg. Mikrobiol.* 2: 164-165.
- ZoBell, C. E. 1943. The effect of solid surfaces upon bacterial activity. *J. Bacteriol.* 46: 39-56.
- Zvyagintsev, D. G. 1962. Some regularities of adsorption of micro-organisms on ion exchange resins. *Microbiology (USSR)* 31: 275-277.

PART F

CHLORINATED HYDROCARBON, MICROPARTICULATE EFFECTS
ON MICROORGANISMS ISOLATED FROM LAKE ERIE

R. M. PFISTER, J. I. FREA, P. R. DUGAN, C. I. RANGLES,
K. ZAEBST, J. DUCHENE, T. McNAIR, AND R. KENNEDY

Abstract

Water samples from the western basin of Lake Erie have been analyzed with regard to the distribution of colloidal microparticles. Size analyses of particulate samples placed on a sucrose density gradient revealed that the most common size particle was in the range of 0.1 μm . Chlorinated hydrocarbon pesticides such as endrin, aldrin, heptachlor and lindane were found in association with these particles and the data suggest that aldrin and heptachlor were found more frequently on the smaller, less dense particles, while lindane was associated with the larger, more dense fractions. Bacteria isolated from these water samples prior to chemical analyses were grown in the presence of clay microparticles freed of pesticides, microparticles containing known amounts of pesticides, and purified pesticides alone. Bacterial growth effects were measured by changes in the turbidity of the medium, total DNA content of the culture and standard plate counts. Results demonstrate that different bacteria in the presence of endrin or aldrin could be affected in different ways. In some cases the organisms were stimulated to produce a cell yield of 4-5 times that of the control cultures. A survey of 151 heterotrophic aerobic bacteria isolated from Lake Erie has shown that 55 were stimulated by aldrin, 54 by endrin and 45 by dieldrin. Forty-six cultures were inhibited by aldrin, 43 by endrin and 43 by dieldrin. Eighteen cultures were stimulated by the three compounds, while 27 cultures were inhibited.

Introduction

The presence and persistence of chlorinated hydrocarbon compounds in the aquatic environment has presented an interesting and potentially dangerous ecological situation. The wide distribution of these recalcitrant organic molecules has raised the question of their long-term effects on our natural environment. The occurrence of these compounds as either molecular aggregates in aqueous solution (4.1 nm or less) or in suspension (up to 0.11 μm) (Bowman, et al., 1960) in the water and the association of these compounds with particulate suspended solids is significant. The interactions and metabolic activities (e.g. growth) of organisms tend to increase at interfaces (Bigger, 1941; ZoBell, 1943; Zvyagintsev, 1962; Pfister, et al., 1968).

It is also known that suspended particles and dissolved chemicals accumulate at interfaces (Riley, 1963; Wood and Oppenheimer, 1962) and that when present represent active sites for adsorption and concentration of materials at the surface. The presence and distribution of pesticides as particulates or in combination with other micro-particulates through adsorption or as stored products in or on minute organisms such as bacteria must be carefully examined and understood. The response of the environment to these compounds must be learned in order to predict or direct corrective measures to be taken to insure environmental safety. Lake Erie is an ideal lake for these studies because of its high rate of man-made organic pollution and its high particulate content.

The purpose of this investigation was to analyze water samples taken from the western basin of Lake Erie (Figure 1) with respect to colloidal particle distribution and size, pesticide content and distribution in the particulate fractions and effects of certain clay and pesticide particulates on bacterial growth.

Methods and Results

Water samples were collected from a depth of 15 feet in Lake Erie in 5 gallon quantities. Heterotrophic bacteria were plated out immediately on plate count agar (PCA). These platings were done as soon as possible upon retrieval of the water sample usually on board the boat. Incubation was carried out at 25°C. After 48 hours, single colonies were picked and restreaked for further experimentation.

The five gallon water sample was passed through a continuous flow high speed centrifuge (Sorvall RC-2B equipped with a Szent-Gorgi continuous flow attachment) at a flow rate of 11 ml per minute and a gravitational force of 27,000. Solid residues from the water sample were weighed and placed on top of a linear gradient of sucrose with a density from 1.0765 to 1.2241. The preparation was centrifuged at 1,500 x G for one hour (Lammers, 1962, 1964, 1967).

Five bands were collected using the tube cutting technique and the microparticles in the bands dialysed against or washed in distilled water by high speed centrifugation (27,000 x G for 30 min.). When

pesticide analysis was to be carried out, the gradient was divided into four bands which were each separately extracted in hexane. The analysis of the hexane extracts for the presence of chlorinated hydrocarbon pesticides was made using an Aerograph 200 gas chromatograph (GLC) equipped with an electron-capture detector (Pfister, Dugan and Frea, 1969).

Particulate Matter in Lake Erie Water Column

Particulates from Lake Erie water samples were evaluated for individual size and size distribution using the Particle Size Analyzer TGZ-3 (Zeiss Co.). This instrument utilizes an illuminated iris diaphragm which can be varied in diameter so that its area can be made equal to the area of a photographed particle (Falcon-Uff, Leverington, 1967).

Lake Erie samples (111A, 111B, 115, 117) were used in this analysis. Samples were prepared for photographing by placing 1-3 micrograms of the particulates on a carbon-coated electron microscope grid. The grids were observed in an Hitachi (HS-7) electron microscope. The fields photographed were both representative of the sample and contained particles which were sufficiently dispersed so that individual particles could be identified (i.e., as little overlapping as possible). On the average, each field contained 100-200 countable particles. Photographic negatives were enlarged so that the smallest particles were not less than 1-2 mm and the largest did not exceed 37.7 mm. Total magnification remained constant throughout a fraction which was to be sized. Photographic positives were made using Kodak Ektamatic paper.

Sizing was accomplished using the exponential-distribution-standard operating condition of the instrument. Each particle was analyzed by placing its photograph over the iris diaphragm, and adjusting the diameter of the circular light spot to equal the area of the particle. If a particle deviated from a circular shape, the light spot was adjusted so that the total area of the protruding portions of the particle became equal to that of the re-entrant areas. The particle was then recorded in a specific counter category in the instrument. A minimum of 1,000 particles per fraction was counted.

Interval centers of the counters were then converted to the actual size of the particles by dividing them by the total magnification. The readings (numbers of particles) obtained on the individual counters were multiplied by the correction factor for exponential counting. The corrected interval centers and the corrected number of particles were then plotted to give a distribution curve. Distribution curves were done on all fractions within a sample (Figure 3-7), and a total distribution curve was plotted for the entire sample (Figure 2).

A student-T test for statistical fitness was also done comparing the samples. The average particle size ranged between 0.14 and 0.24 μ . The absolute particle range, however, was as low as .029 μ and as high as 7.90 μ (Table 2).

In the individual fractions a large peak in the number of particles usually occurred in the 0.08 to 0.2 μ range. This range accounted for, on the average, 50-55% of the total particles counted.

The T-statistic information (Table 3) shows that all three samples were significantly different.

Pesticide Distribution on Microparticulates

Examination of particulates from five water samples for the presence of chlorinated hydrocarbons showed that there was a distribution of these compounds through the gradient. In these five cases, the gradient was divided into four sections, each of which was analyzed separately. With only five complete samples, no real significance could be attached to the distribution patterns with respect to each original water sample. When the total amount (nanograms) of each pesticide was calculated and an indication of the fraction from which it was isolated was made, a distribution pattern emerges. Figure 8 shows the chlorinated hydrocarbons dieldrin; p,p'-DDE; endrin; heptachlor; aldrin; lindane; and o,p'-DDD and their cumulative distribution (from five samples) in the four fractions from the sucrose gradient. In the case of endrin and aldrin, the largest amounts were recovered from fractions 1 and 3 when compared to fractions 2 and 4. Very little lindane was recovered in fractions 1 and 2 (about 125 nanograms) while 680 nanograms were recovered in fractions 3 and 4. The o,p'-DDD appeared to be more evenly divided with slightly more in fraction 4. Dieldrin and p,p'-DDD were also more evenly divided in all fractions except for fraction 3. The lowered amount in fraction 3 could also be seen in the o,p'-DDD and the heptachlor samples where most of the pesticide remained in fractions 1 and 2.

Interaction of Pesticides and Microparticles on Bacterial Growth

In this study we have exposed a culture of a Pseudomonas species isolated from Lake Erie (OSU isolate #501) as previously described (Pfister, Dugan and Frea, 1968) to various concentrations of the chlorinated hydrocarbon pesticides endrin and p, p'-DDE. These compounds were placed in suspension using double distilled hexane or were adsorbed to the clay kaolinite after it had been preextracted and washed in hexane and acetone. In order to adsorb pesticides onto the clay, 0.3 g of kaolinite was suspended in 100 ml double-distilled demineralized water. One ml of a stock solution (endrin, 0.1 g/ml or 0.001 mg/ml; p,p'-DDE, 0.01 mg/ml or 0.001 mg/ml) of pesticide was added and left on a rotary shaker for 24 hours at 21°C. The clay was then washed three times in double-distilled, demineralized water to remove any unadsorbed pesticide aggregates. Samples of the extracts and the presence of the hydrocarbons on the clay were examined using the GLC technique. The final concentration of endrin in the medium was 3.7 µg/ml (conc) and 3.7×10^{-4} µg/ml (dil) of p,p'-DDE was 3.7×10^{-3} µg/ml (conc) and 3.7×10^{-4} µg/ml. The bacteria were cultured in a defined medium as shown in Table 1. All cultures were grown on a rotary shaker at 21°C.

Growth was determined through measurement of turbidity (Klett-Summerson Colorimeter equipped with a 540 NM filter) every 24 hours and with a diphenylamine assay for analyses of total deoxyribose nucleic acid (DNA) at completion of the experiment. (Dische, 1955). All experiments were repeated in triplicate.

In order to relate DNA content to dry cell weight, cells were washed and suspended in distilled water and measured amounts pipetted into tared weighing cups. An equal amount was put in a glass centrifuge tube and the DNA content determined with diphenylamine. The weighing cups were dried in an oven at 100°C and weighed at 24 hour intervals until the weight remained constant. DNA content and dry cell weight were then plotted relative to an untreated control (Figs. 9,10).

In order to relate the increase in turbidity observed during the experiments and the increase in DNA content with an increase in cell number, viable plate counts were also made. These were done every 24 hours by dilution in sterile water blanks followed by plating on PCA (Difco). Plates were incubated for 48 hours at 21°C and counted at the appropriate dilution. Results of these counts always showed a consistent increase in viable cells which followed substantially the increases in turbidity and DNA.

The growth rate of a Pseudomonas sp. in defined medium was found to be greatly enhanced by the addition of 0.04 grams of kaolinite per 35 milliliters. With the addition of 0.13 ml double-distilled hexane, an increase in growth was also observed. In the case where pesticides were added in a non-adsorbed aggregated form, this acted as a control since the pesticides were dissolved in hexane. A sample of each pesticide was adsorbed on kaolinite and the effect tested on growth. Concentrated p,p'-DDE enhanced growth both by itself and while adsorbed to the kaolinite. Dilute p,p'-DDE enhanced growth slightly while adsorbed to clay, but caused a decrease in growth when by itself. The amounts of growth measured by turbidity and DNA in each experiment were closely parallel throughout

the experiment (Figure 9). Concentrated endrin seemed to inhibit growth while dilute endrin caused an increase (Figure 10).

Survey of Lake Erie Heterotrophic Bacteria in Response to Pesticides

A survey of the response of 151 heterotrophic aerobic bacteria isolated over a two year period from Lake Erie to the presence of three chlorinated hydrocarbon pesticides has been carried out. Aldrin, endrin and dieldrin were placed in 9.9 ml of the medium previously described to a final concentration of 1.0 $\mu\text{g/ml}$. Acetone was used to dissolve the hydrocarbons and 0.1 ml of the stock solution was added to each growth flask. Incubation was carried out at $25^{\circ} \pm 2^{\circ}$ for one week in quiescent culture. Five growth flasks in replicates were prepared and samples monitored for absorbancy compared to control flasks in a Coleman autoset spectrophotometer. The results of these analyses indicated that many of these cultures were changed in their growth patterns and were in some cases significantly stimulated or depressed in the presence of these compounds. Of the 151 cultures examined, 55 were stimulated by aldrin; 54 by endrin and 45 by dieldrin. Examination of the results showed that 46 cultures were inhibited by aldrin; 43 by endrin and 43 by dieldrin. In many instances, cultures were effected by more than one pesticide. All three pesticides were stimulatory to 18 of the cultures, while 27 were inhibited by them.

Discussion

These data show (Figure 3-7) that in the sample tested from Lake Erie (Zone A, Figure 1) there was a distinct distribution of micro-particles which could be separated by density gradient centrifugation. Figure 2 shows the population of particles from sample 111B prior to having been centrifuged in the sucrose gradient. The greatest number of particles are found in the size range of 0.05-0.2 microns (colloidal size). This has been the case in every sample examined to date and can be seen in the results shown in Table 2. In sample 111A taken on 3/21/69 in Zone A, there was an average particle size of 0.24 microns with an absolute range of 0.029 to 7.9 microns. Each of the other samples 111B, 115 and 117 reflect essentially the same result which suggests that the majority of particles are indeed in the small size range.

Additionally, it can be seen that the particles from Zone A and Zone B taken on the same date only one hour apart have significantly different size (Table 3). The particles from Zone B, which is east of Zone A are smaller in average size. Lake Erie is known to become less turbid toward the eastern end, so it is not surprising to find smaller particles in Zone B. When samples were taken in Zone A or B on different dates, under different weather conditions (e.g., wind direction, velocity, and temperature differences) the particle sizes (sample 115, 117) were also different (Table 3). Since Lake Erie is shallow, especially in the western basin, altered wind direction and velocity severely affects the particulate content of the water. It can be generally concluded from these data that the majority of the particles are colloidal in size and

that they do vary with either location or time of sampling. It will be of future interest to learn the associations or interactions of microorganisms with the varying particle content. This is of importance because the particles can be separated in sucrose gradients (Figures 3-7) and have been found to demonstrate a varied chemistry. In this study (figure 8) we have concluded that different hydrocarbon pesticides may be adsorbed or absorbed on or in different particles. Whether or not this is particle-specific is not presently known, but the data suggests that certain compounds can be found in association with lighter, smaller or more dense or larger particles. This finding is interesting in light of the fact that different particle sizes may be found in the lake as previously discussed. It is obvious then, that the consideration of not only the weather, but the type of particle input into the lake becomes significant with respect to involvement in the microecology.

The presence of recalcitrant organic pollutants is increasing in the natural environment and it has been generally conceded that the problems are arising because of the inability of microorganisms to degrade these pesticides, at least not at rates which prevent accumulation and serious water or soil pollution. Examination of Figures 9 and 10 raise still another concern as yet unexplored. Microparticles isolated from Lake Erie do effect the growth of the cultures isolated from the lake. The experiment shown here (Figure 9) demonstrates that *p,p'*-DDE in low concentration does stimulate a bacterial culture. The clay microparticles themselves stimulated the growth of this culture, and the combination

of microparticles containing pesticide was most satisfactory in enhancing growth. The compound endrin and the same culture had less pronounced effects, but even there, stimulation of the cells by the washed clay and the pesticide adsorbed clay was detected.

The association of these chlorinated hydrocarbon chemicals with certain components of the colloidal particle system in Lake Erie appears to be a significant one and deserves future study. Whether or not these effects are widespread in the microecology also needs to be evaluated. We have examined 151 heterotrophic bacteria isolated over a 2 year period in the western basin of Lake Erie. The results of this study suggest that a large percentage of these types are affected by the presence of such recalcitrant molecules whether they utilize the compounds in some way or not. The important point is that they may have an effect on the microbial microenvironment and must be considered as potentially dangerous long-term interlopers. As pesticide-laden sediments accumulate in a lake such as Erie, and it becomes more eutrophic, these effects will become more prominent. How these reactions effect the primary productivity of any body of water is of prime importance.

REFERENCES

- Bigger, J. W. and J. H. Nelson. 1941. The growth of coliform bacilli in distilled water. *J. Pathol. Bacteriol.* 53: 189-206.
- Bowman, M. S., F. Acree, Jr. and M. K. Corbett. 1960. *J. Agr. Food Chem.* 8, 406.
- Dische, Z. 1955. Color reactions of nucleic acid components. In *The Nucleic Acids, Vol. I.* E. Chargoff and J. N. Davidson (Ed.) 285-305. Academic Press, N. Y.
- Falcon-Uff, P., and K. F. Leverington. 1967. The determination of silver halide crystal size distribution in photographic emulsions with the Zeiss-Endter particle size analyzer. In *Particle Size Analysis Proc. of the Soc. for Analytical Chem.* W. Hiffer and Sons, Ltd., Oxford.
- Lammers, W. T. 1962. Density gradient separation of plankton and clay from river water. *Limnology and Oceanography* 7: 224-229.
- _____ 1964. Density gradient separation of plankton and clay from river water: Theory and applications. *Verhandl. Intern. Verein. Limnology* 15: 1021-1028.
- _____ 1967. Biophysical Limnology - Separation of suspended and colloidal particles from natural water. *Environmental Science and Technology*, Vol. I, No. 1. Jan. 1967. Pg. 52-57.
- Pfister, R. M., P. R. Dugan and J. I. Frea. 1968. Particulate fractions in water and the relationship to aquatic microflora. *Proc. 11th Conf. Great Lakes Res. International Assoc. Great Lakes Res.* 111-116.

REFERENCES (CONT'D)

- Pfister, R. M., P. R. Dugan and J. I. Frea. 1969. Microparticulates: Isolation from water and identification of associated chlorinated pesticides. *Science*, Vol. 166, p. 878-879.
- Riley, G. A. 1963. Organic aggregates in seawater and the dynamics of their formation and utilization. *Limnology and Oceanography* 8: 372-381.
- Wood, E. J. F., and C. H. Oppenheimer. 1962. Note on fluorescence microscopy in marine microbiology. *Z. F. Allg. Mikrobiol.* 2: 164-165.
- ZoBell, C. E. 1943. The effect of solid surfaces upon bacterial activity. *J. Bacteriol.* 46: 39-56.
- Zvyagintsev, D. G. 1962. Some regularities of adsorption of microorganisms on ion exchange resins. *Microbiology (USSR)* 31: 275-277.

TABLE 1

	g/l
arginine	1.0
dextrose	10.0
$MgSO_4 \cdot 7H_2O$	0.4
K_2HPO_4	4.0
KH_2PO_4	2.0
xanthine	0.005
guanine	0.005
adenine	0.005
uracil	0.005
riboflavin	0.005
nicotinic acid	0.025
B-alanine	0.025
Pyridoxine	0.010
Folic Acid	0.00005
Biotin	0.0001
PABA	0.005
Vitamin B ₁₂	0.000003

TABLE 2

<u>Sample</u>	<u>Date</u>	<u>Location</u>	<u>Corrected No. Particles Counted</u>	<u>Average Particle Size</u>	<u>Absolute Particle Range</u>
111-A	3/21/69	Zone A	21,070	0.24 μ	.029 μ to 7.9 μ
111-B	3/21/69	Zone B	11,252	0.14 μ	.039 μ to 1.5 μ
115	5/16/69	Zone B	9,433	0.22 μ	.061 μ to 2.60 μ
117	5/29/69	Zone A	6,033.9	0.19 μ	0.062 μ to 2.60 μ

TABLE 3

<u>Sample</u>	<u>Sample</u>	<u>T-Statistic</u>
111-A	111-B	29.60
111-A	115	5.15
111-A	117	10.35
111-B	115	39.20
111-B	117	29.86
115	117	10.36

FIGURE LEGENDS

Fig. 1. Map of western basin of Lake Erie showing areas of study (Zone A and Zone B).

Fig. 2. Total size distribution of microparticles in sample 111B before gradient separation.

Fig. 3. Fraction of linear sucrose gradient (top) showing the size distribution of microparticles in 111B.

Fig. 4. Fraction 2 of sucrose gradient (second from top) showing the size distribution of microparticles in 111B.

Fig. 5. Fraction 3 of sucrose gradient (third from top) showing the size distribution of microparticles in 111B.

Fig. 6. Fraction 4 of sucrose gradient (fourth from top) showing the size distribution of microparticles in 111B.

Fig. 7. Fraction 5 of sucrose gradient (bottom) showing the size distribution of microparticles in 111B.

Fig. 8. Histogram showing the association of the pesticides dieldrin, p,p'-DDE, endrin, heptachlor, aldrin, lindane, and o,p'-DDD with each of four fractions from linear sucrose gradients of five different lake water samples.

Fig. 9. Graph plotted as relative difference in growth of a Pseudomonas sp? from Lake Erie grown in the presence of p,p'-DDE either in pure chemical form or adsorbed to the clay kaolinite.

Fig. 10. Graph plotted as relative difference in growth of Pseudomonas sp? from Lake Erie grown in the presence of endrin either in pure chemical form or adsorbed to the clay kaolinite.

FIGURE LEGENDS (CONT'D)

Table 1. Defined medium used to culture the heterotrophic bacteria isolated from Lake Erie during the experiments.

Table 2. Shows the four water samples analyzed in this study, their date, location, particle count, average size and absolute particle range.

Table 3. An analysis of the four samples using the Student-T statistic.

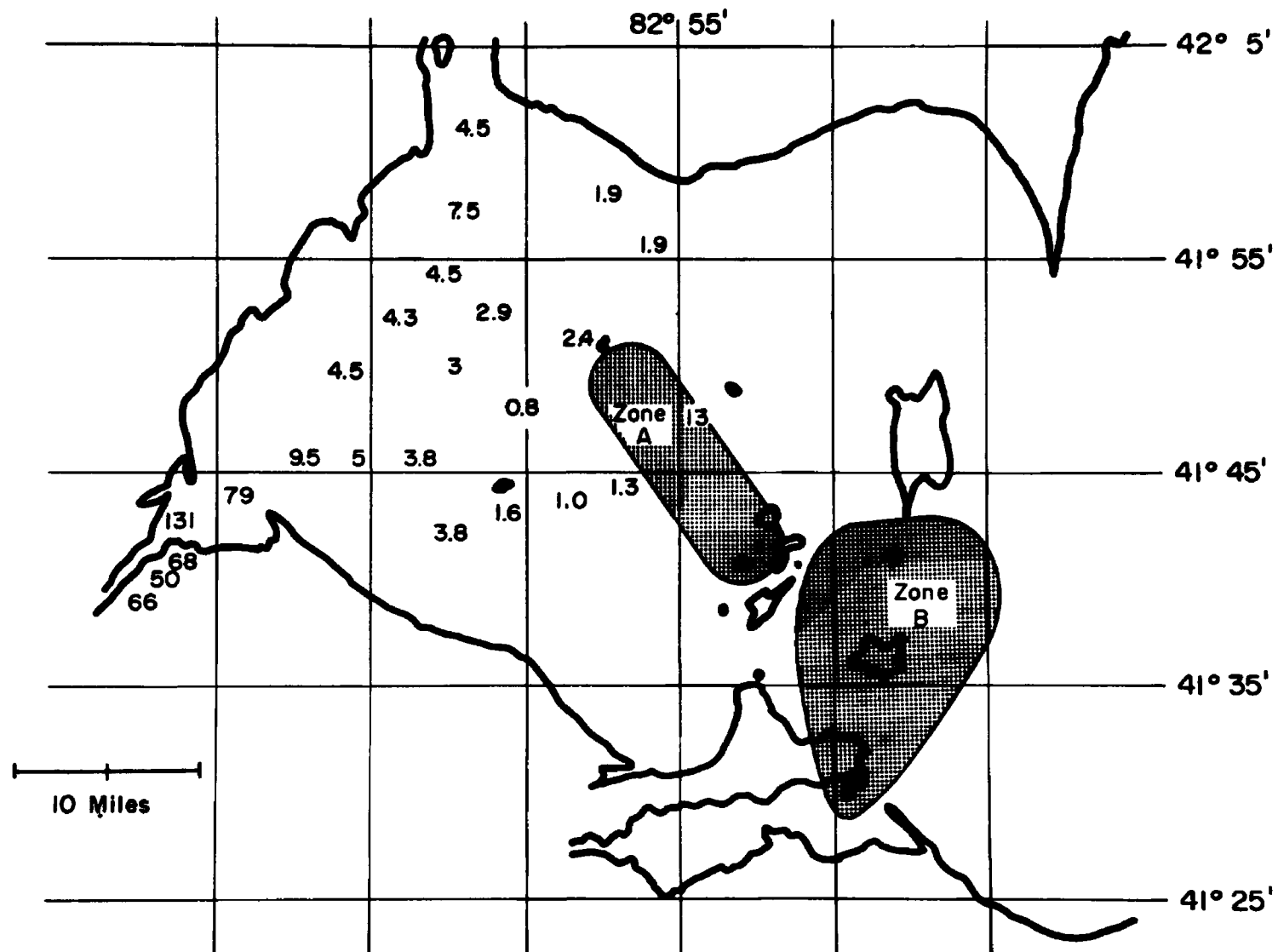


Figure 1

Map of Western Basin of Lake Erie, showing areas of study.
(Zone A and Zone B).

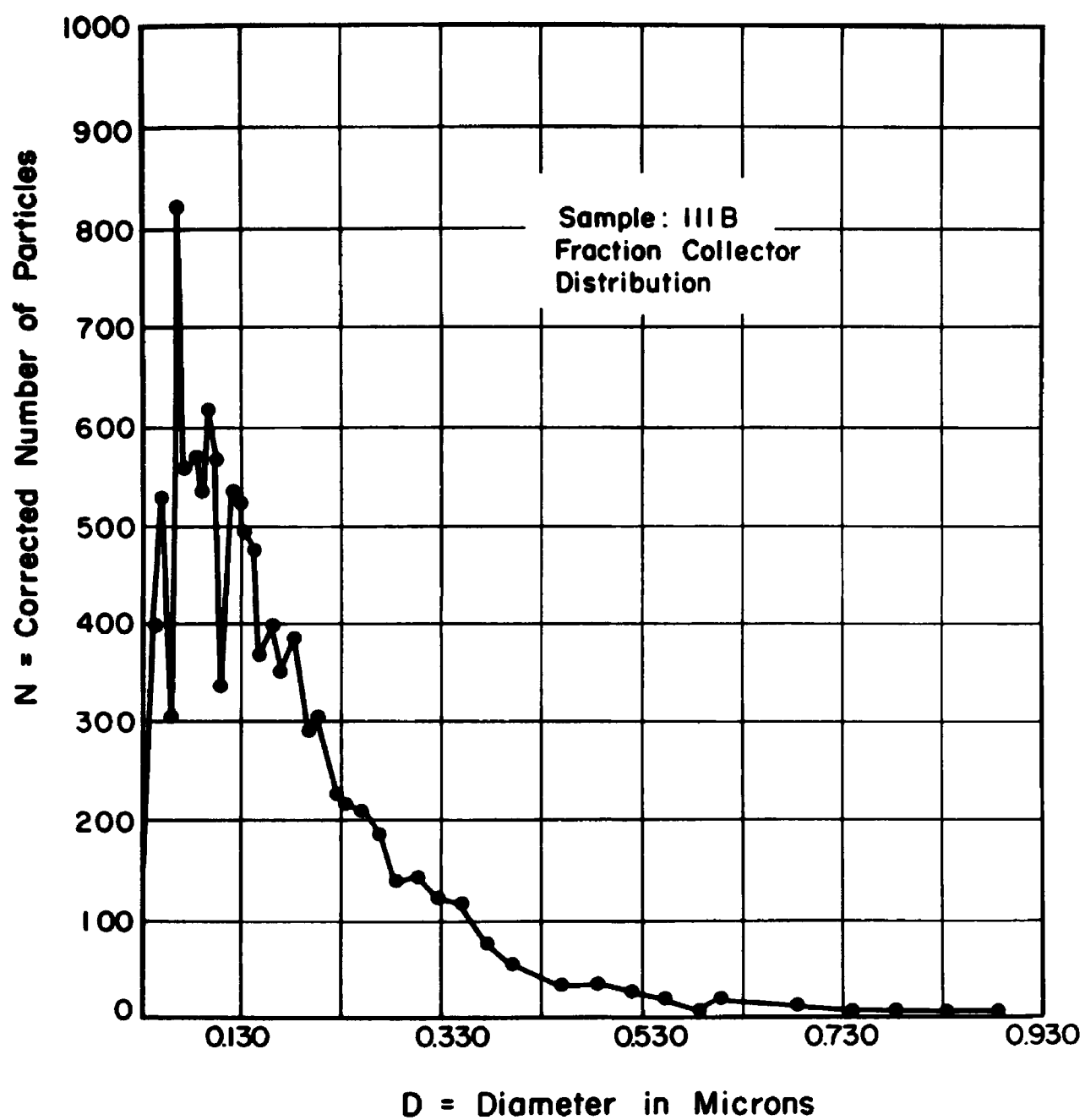


Figure 2

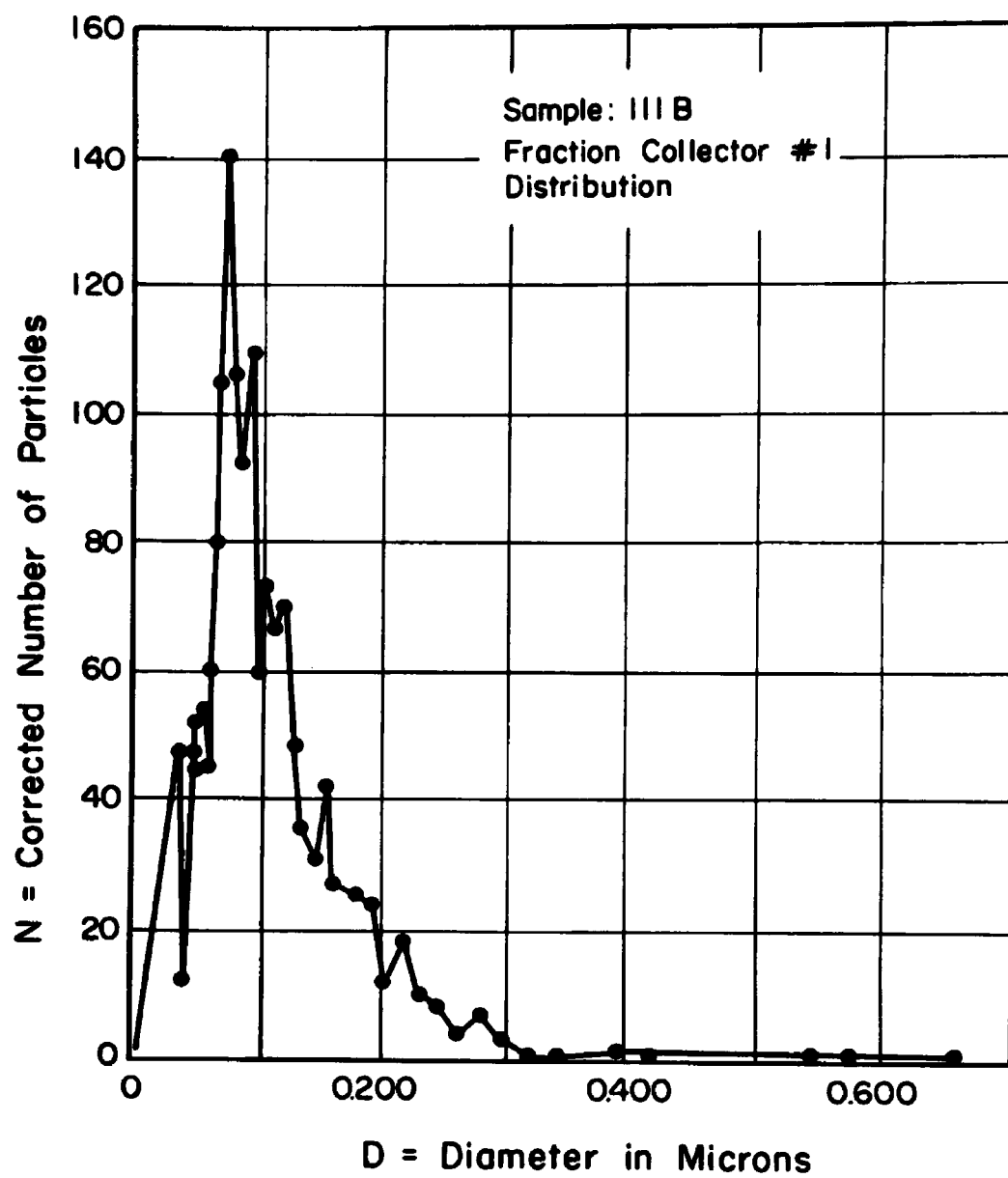


Figure 3

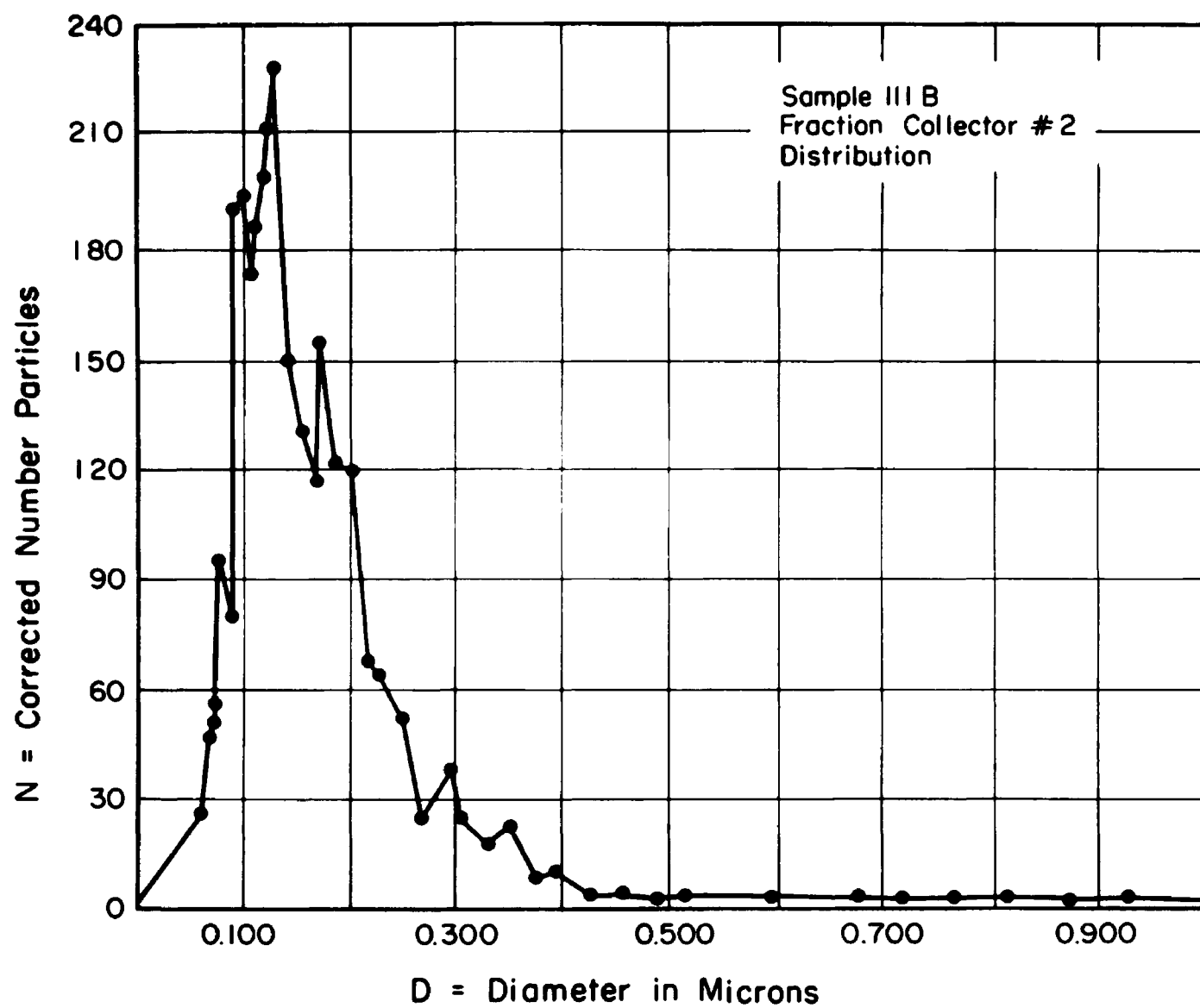


Figure 4

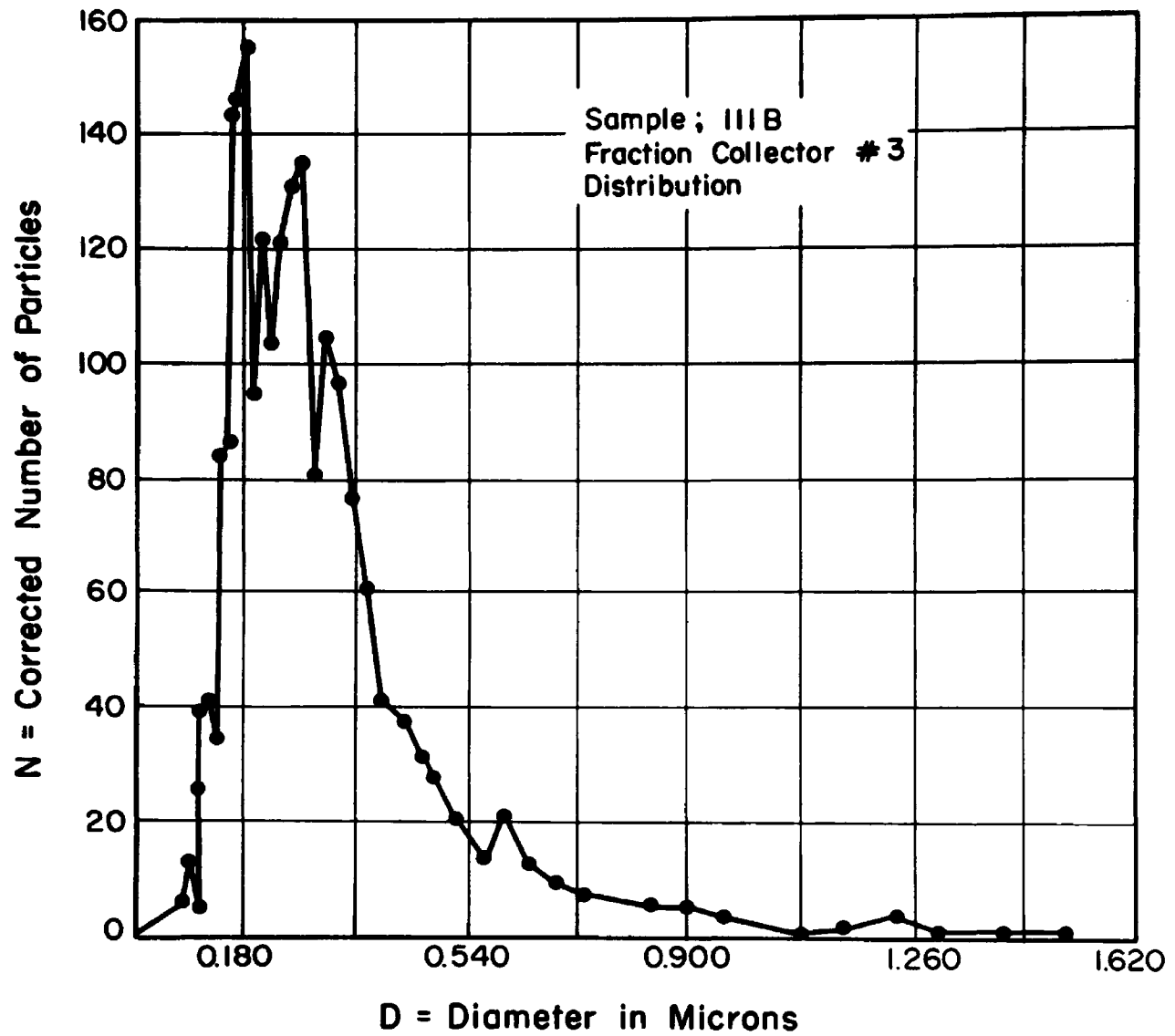


Figure 5

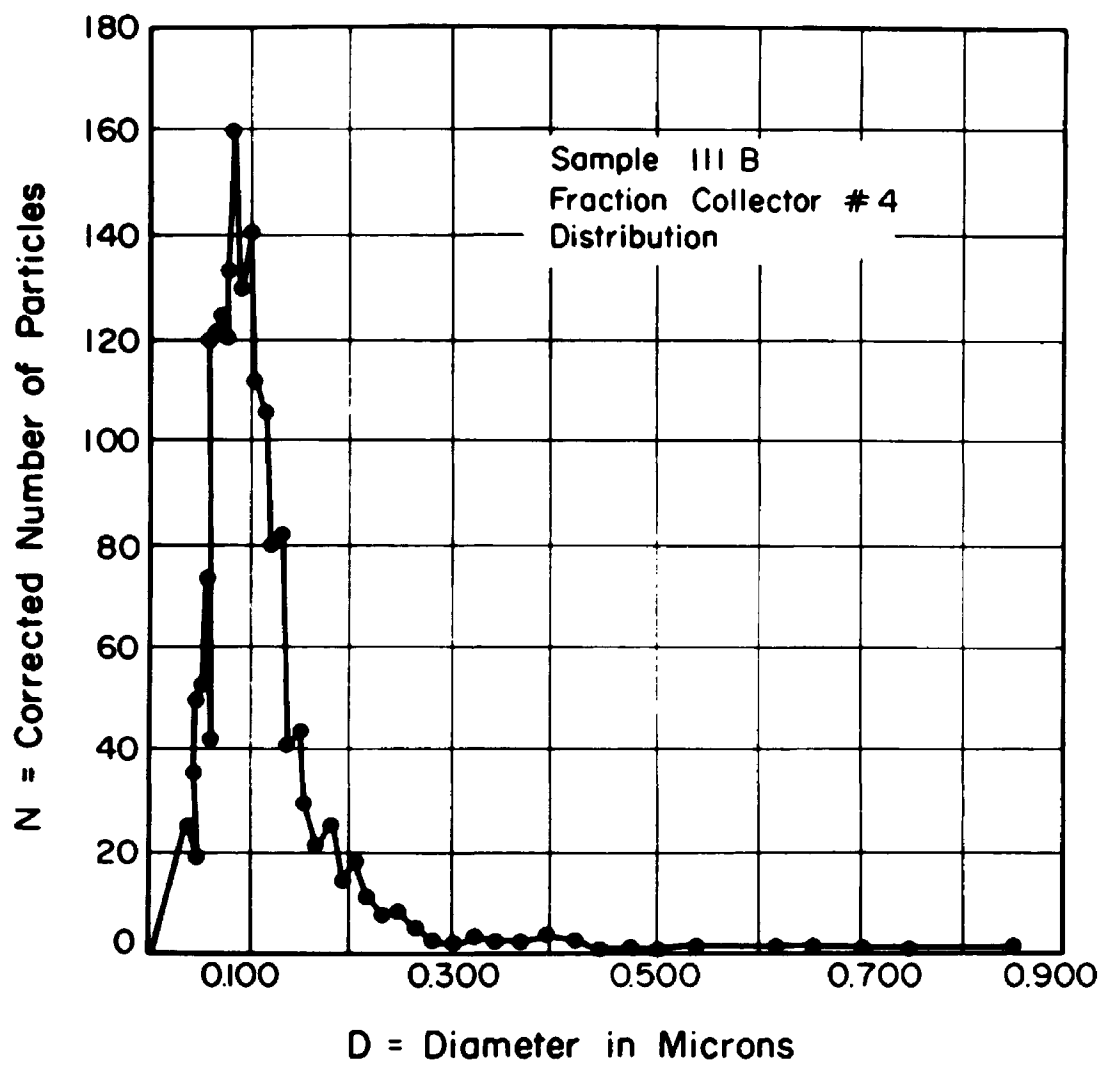


Figure 6

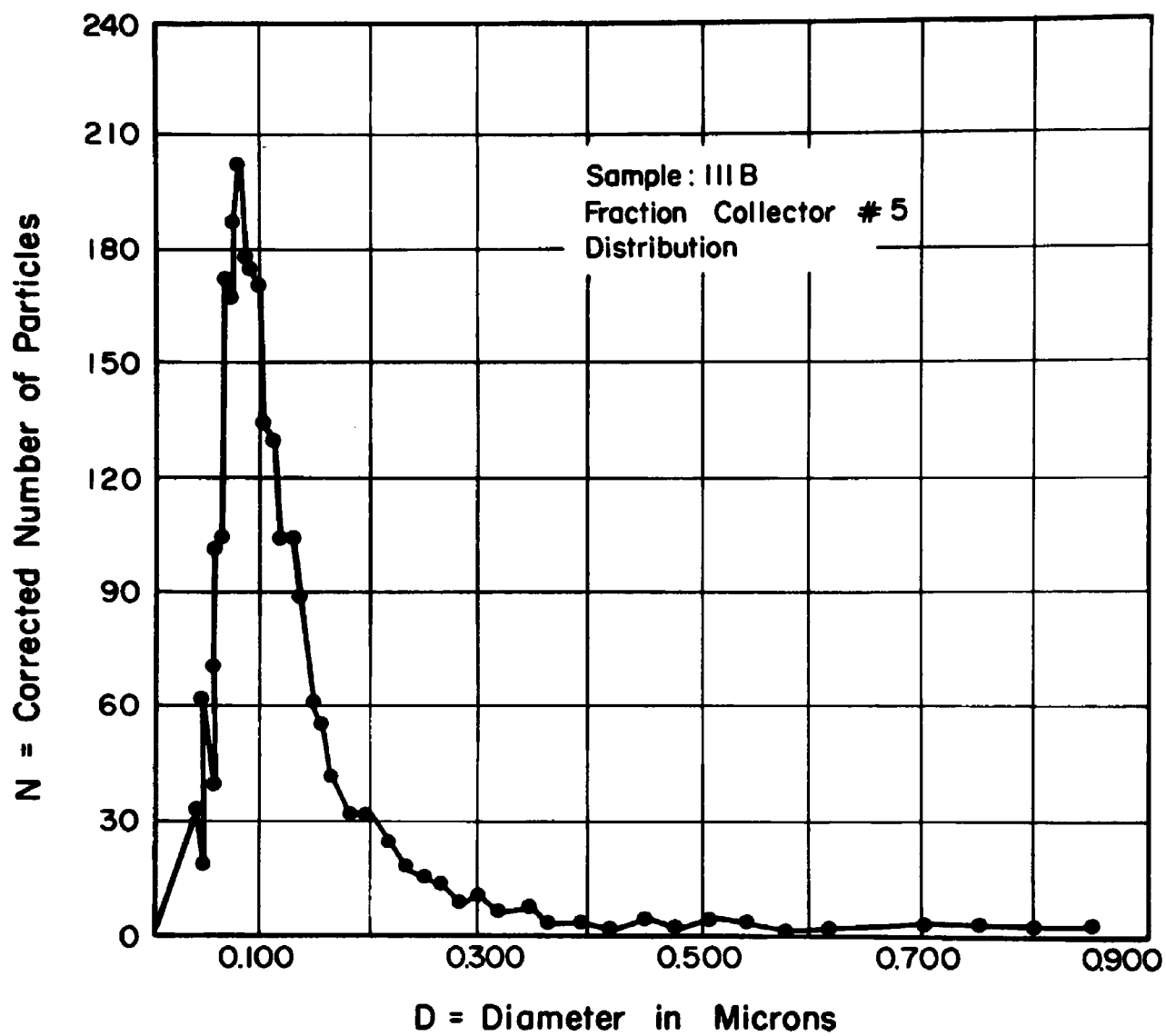


Figure 7

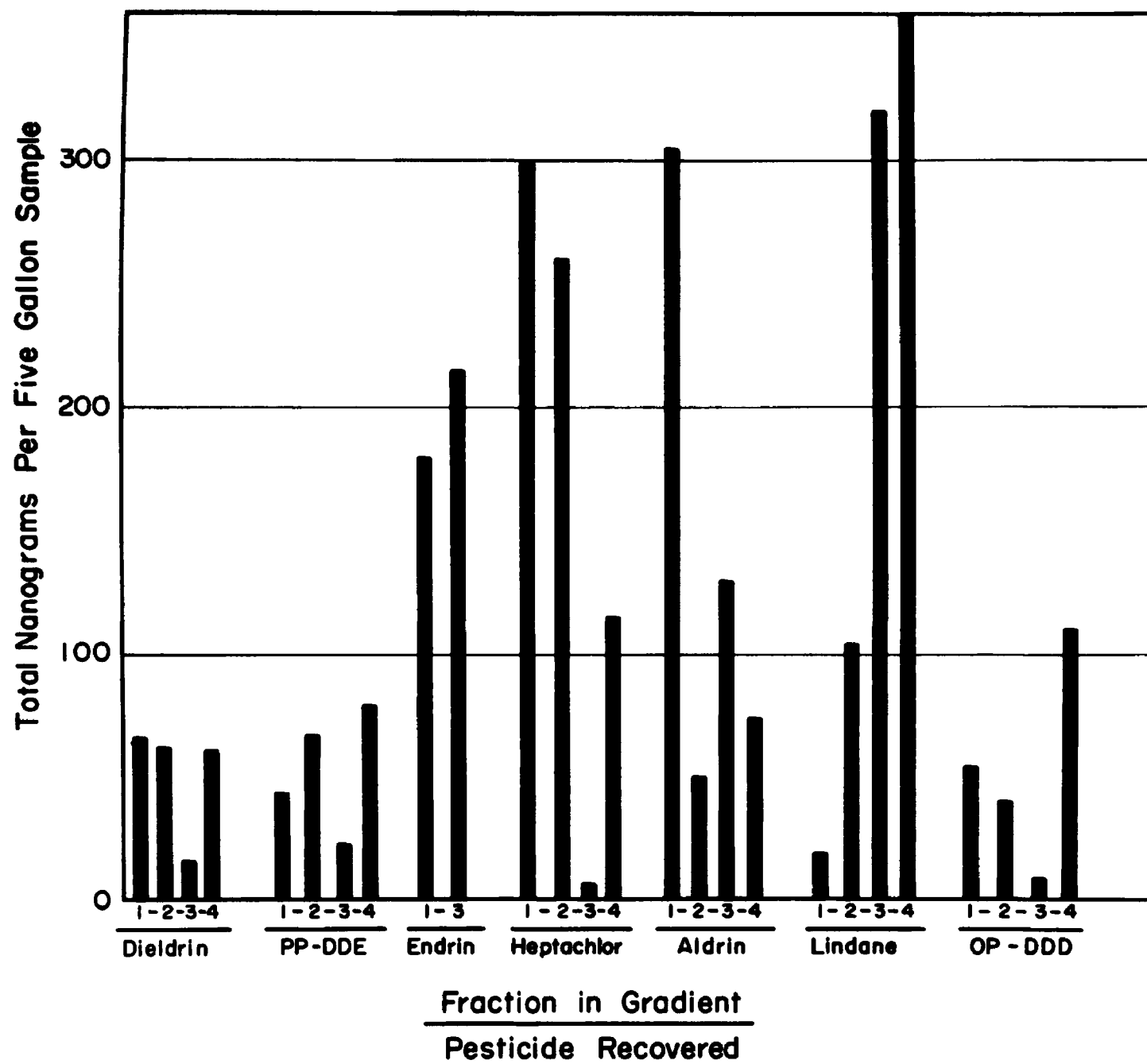


Figure 8

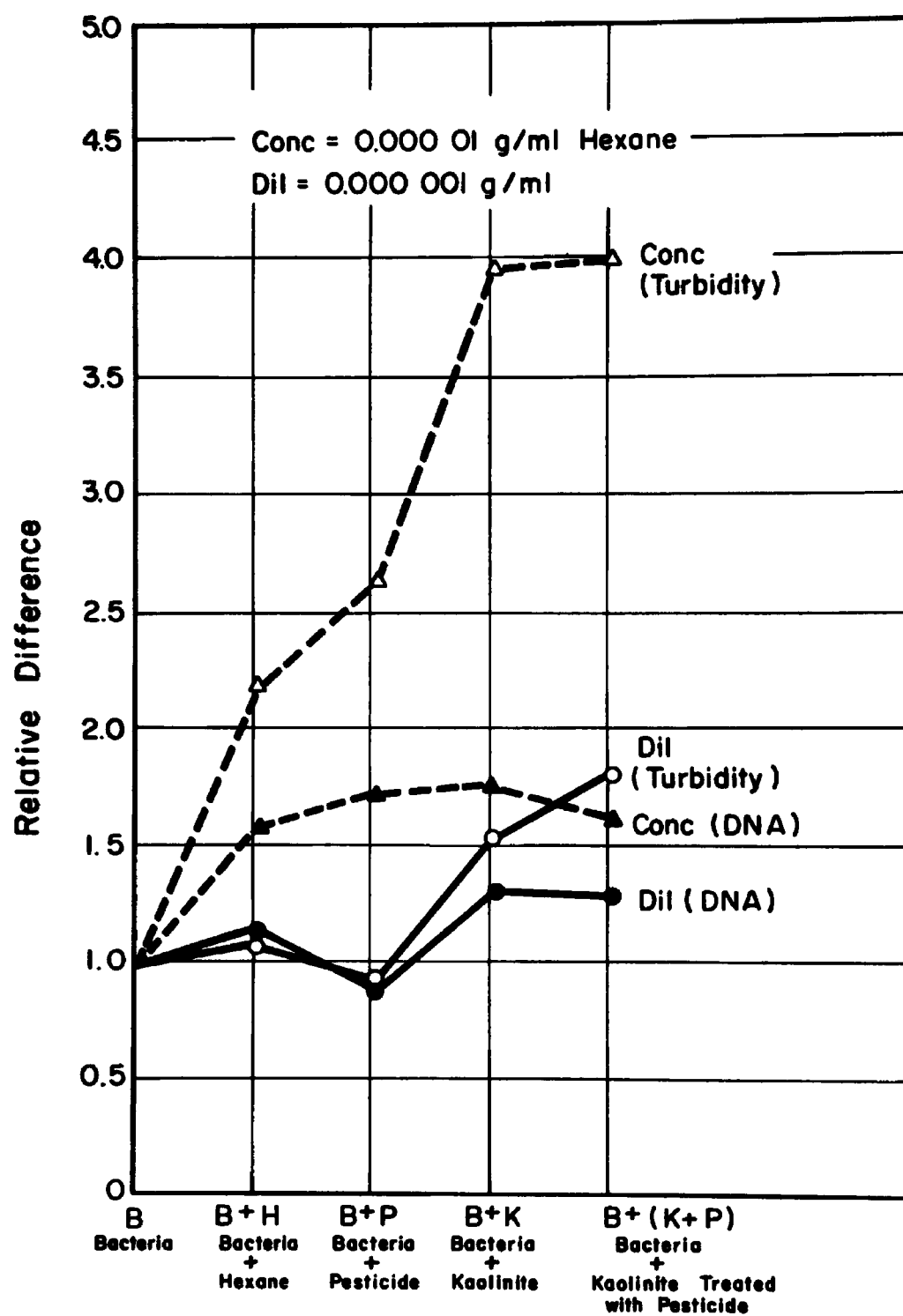


Figure 9

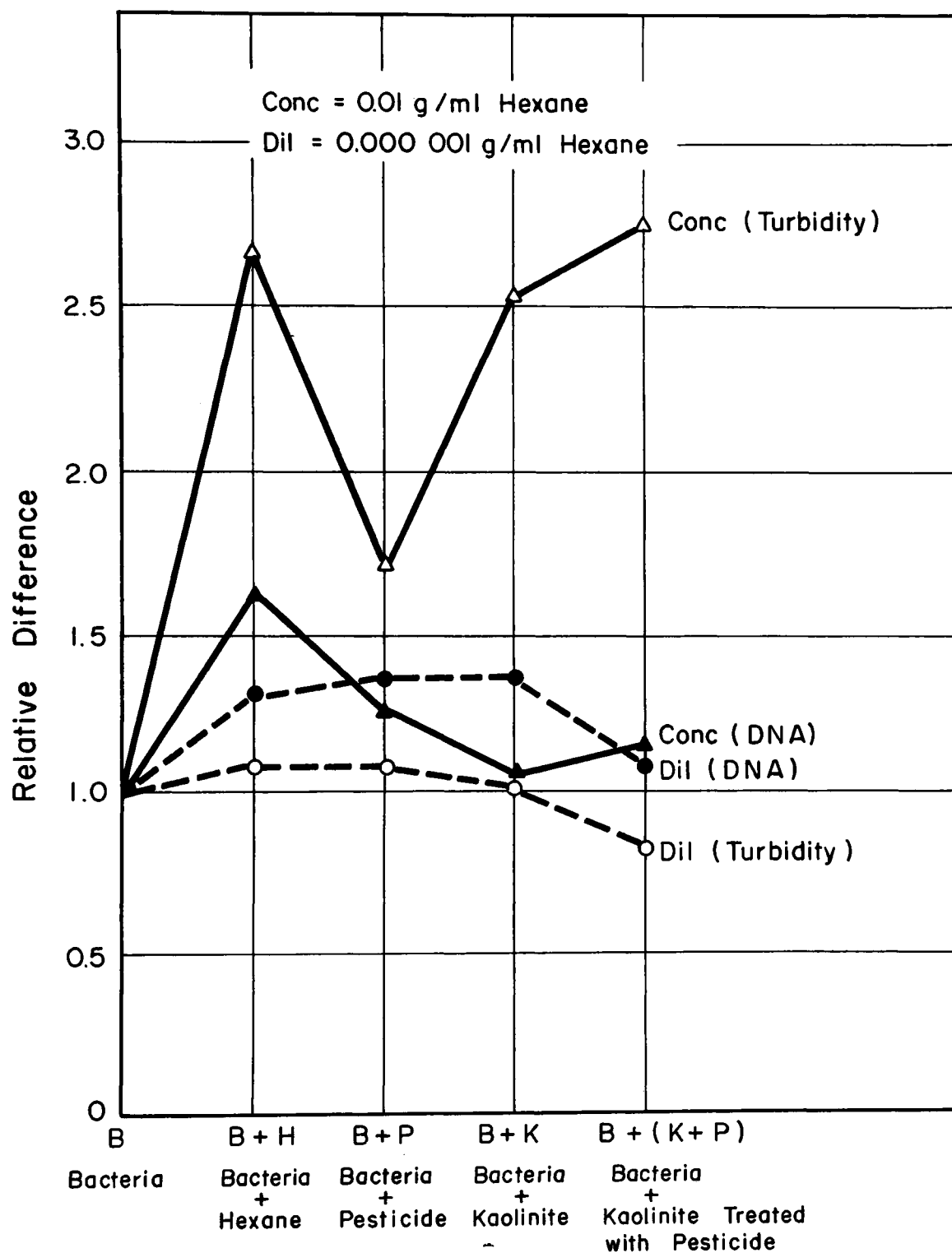


Figure 10